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Effect of Different Bacterial Fertilizers on Soil Carbon Mineralization

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ABSTRACT

In this study, aimed to investigate the effect of bacterial fertilizer: A (*Bacillus* spp., *Trichoderma* spp.), B (*Azorhizobium*, *Azotobacter* and *Azospirillum*) and C (*Azotobacter* spp., *Bacillus* spp. and *Pseudomonas putida*) on soil carbon mineralization. On the application of A, B and C bacterial fertilizers on the sterilized control soil, whose initial carbon mineralization rates is 1.1%, mineralization rates of 5.12%, 3.54%, and 10.78% were respectively recorded. According to these results, it was observed that the application of bacterial fertilizer increased the carbon mineralization rate of the sterilized control soil by 365.45%, 221.82% and 880%, respectively. A carbon mineralization rate of 7.03%, 6.15% and 12.95% was recorded in the non-sterilized soil sample whose initial carbon mineralization rate is 5.1%, thereby increasing the mineralization rate by 25.31%, 9.63% and 130.84%. The application of the bacterial fertilizer to the soil was found to increase the soil carbon mineralization rate. It is recommended to incorporate bacterial fertilizers with CO_2-sequestering materials, such as biochar, to mitigate the fluctuations in the natural balance due to carbon release.

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Farklı Bakteriyel Gübrelerin Toprak Karbon Mineralizasyonu Üzerine Etkisi

ÖZET

Bu çalışmada, farklı içerikteki A (*Bacillus spp., Trichoderma spp.*), B (*Azorhizobium, Azotobacter ve Azospirillum*) ve C (*Azotobacter spp., Bacillus spp.* ve *Pseudomonas putida*) bakteriyel gübrelerinin toprak karbon mineralizasyonu üzerindeki etkisini araştırmayı amaçlandı. Karbon mineralizasyon oranı %1,1 olan steril edilmiş kontrol toprağına A, B ve C bakteri gübreleri uygulanmış ve karbon mineralizasyon oranları sırasıyla %5,12, %3,54 ve %10,78 olarak ölçülmüştür. Bu sonuçlara göre bakteri gübresi uygulamanın steril edilmiş kontrol toprağının karbon mineralizasyon oranını sırasıyla %365,45, %221,82 ve %880 oranında arttığı görülmüştür. Karbon mineralizasyon oranı %5,1 olan steril edilmemiş toprak örneğinde de A, B ve C bakteri gübre uygulamasının karbon mineralizasyon oranları sırasıyla %7,03, %6,15 ve %2,95 olarak ölçülmüş olup bakteri gübre uygulamasının karbon mineralizasyon oranını sırasıyla %25,31, %9,63 ve %130,84 arttırdığı belirlenmiştir. Çalışmanın sonunda bakteriyel gübre uygulamasının toprak örneklerinde karbon mineralleşmesini artırdığı sonucuna varılmıştır. Bu artışın doğal dengeyi bozmasını önlemek için bakteri gübreleri uygulanırken biochar gibi karbon bağlayıcıların da verilmesinin uygun olacağı düşünülmüştür.

Mikrobiyoloji

Araştırma Makalesi

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Anahtar Kelimeler
Bakteriyel gübre
Karbon mineralizasyonu
Bitki ekolojisi
Metagenom

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INTRODUCTION

Biofertilizer contains live microbes that colonizing the rhizosphere or endosphere of plants, induce growth by increasing the uptake of essential nutrients by the host plant. These microorganisms are known to regulate the natural nutrient cycle and the production of soil organic matter (Rokhzadi and Toashih, 2011, Ekici et al., 2022). Despite the many benefits derived from microbial biotechnology application in agriculture, there are challenges that need to be addressed and

opportunities that need to be explored (Prasad et al., 2019)

Soil microbial biomass, a sensitive indicator of soil quality, is of immense importance in agriculture. Organic matter decomposition is enhanced by bacteria which by extension impact significantly on nutrient cycle in the ecosystem (Maestre et al., 2015; Carini et al., 2016; Tedersoo, 2017). Intensive agricultural practices which involves the utilization of synthetic fertilizer have been found to impact on the environment negatively (Barros de Medeiros & Lopes, 2006; Mahdi et al., 2010; Xiang et al., 2012). Plant growth is strongly influenced by environmental factors such as temperature, water, and nutrient availability. By increasing the availability of nutrients for plant utilization in the soil, plant growth promoting bacteria (PGPB) enhances plant growth (Prasad et al., 2019).

Intensive soil management practices lead to a decline in soil microbial carbon fixation (Silva et al., 2010). Soil microbial activity is impacted by the complexity, quality, and quantity of available organic matter in the soil. Hence, soil microbial biomass carbon (SMBC) is likely to be sensitive to introducting a new plant cover (Belo et al., 2012). Soil microbiological property is usually improved through the application of biofertilizer. Unlike soil that was not treated with biofertilizer or is left to fallow, soil treated with biofertilizers was found to exhibit higher SMBC and lower CO_2 loss. Biofertilizer application to soil improves the microbiological quality of the soil (Ascari et al., 2019).

Other downsides of using synthetic fertilizers in agriculture include a decline in soil fertility, water pollution, eutrophication, biodiversity loss and soil acidification (Tomer et al., 2016; Kourgialas et al., 2017; Mahanty et al., 2017).

To preclude the negative impact on the ecosystem arising due to the utilization of synthetic fertilizer in agriculture, over the last decades, efforts have been channeled towards the utilization of eco-friendly biofertilizer as a substitute for synthetic fertilizer biofertilizer (Liu and Lal, 2015; Davarpanah et al., 2016; Mikhak et al., 2017). One of the advantages of biofertilizers over synthetic ones is their ability to minimize leaching (Subbarao et al., 2013; Malusa et al., 2016), plant growth promoting rhizobacteria (PGPB) are usually utilized as biofertilizers; these organisms commonly establish themselves in the rhizosphere of plants where they execute a variety of functions that are of benefit to the plant through direct or indirect plant growth promotion (Zhang et al., 2014). Direct and indirect plant growth promotion is achieved through the following: phosphate solubilization, auxin secretion, nitrogen fixation, pathogen antagonists through competition and antibiotics secretion (Ahmad et al., 2012; Mohite, 2013; Yin et al., 2015; Puri et al., 2016).

Alcaligenes, Azospirillum, Bacillus cereus, B subtilis., B. circulans, *Enterobacter, Flavobacterium, Klebsiella, Pseudomonas gladioli, P. putida, P. fluorescens, Serratia* are some of the bacteria commonly utilized in plant growth promotion due to their ability to produce plant growth regulaors such as abscisic acid, auxins, ethylene, gibberellins and cytokinins (Arshad & Frankenberger, 1997). Plant growth promotion by *Bacillus amyloliquefaciens, B. cereus and B. subtilis* was the most effective (Francis et al., 2010).

Increase in crop yield is achieved through soil quality improvement, thereby mitigating the adverse effect of climate change (Shrestha et al., 2013). CO_2 emissions in agriculture include total microbial respiration in plant rhizosphere and soil (Rochette et al., 1999). One of the principal fluxes in the global carbon cycle is CO_2 emissions from soil. Atmospheric CO_2 concentration could be strongly affected by a slight change in the magnitude of soil CO_2 flux (Schlesinger and Andrews, 2000). To understand the changing global carbon cycle, knowledge of the factors modulating soil CO_2 flux and carbon sequestration becomes pertinent. Soil CO_2 flux is affected by agricultural practices that modify soil conditions. Microbial activity in soil is affected by soil characteristics, soil organic matter (SOM) and decomposition, which leads to the evolution of CO_2 from Carbon synthesized by plants (Franzluebbers et al., 1995). Carbon mineralization has been proposed to serve as a device for detecting changes in soil microbial ecology resulting from the interactions between inoculant and indigenous microbial populations of soil.

In-vitro effect of A (*Bacillus* spp., *Trichoderma* spp.), B (*Azorhizobium*, *Azotobacter* and *Azospirillum*) and C (*Azotobacter* spp., *Bacillus* spp. and *Pseudomonas putida*) bacterial fertilizers inoculation on carbon mineralization in the soil as well as interactions between the bacteria in the fertilizers and the soil microbial community was investigated.

MATERIAL and METHOD

Sampling Method

Soil samples were collected from uncultivated fields in Datça-Knidos County (30°43'31" N, 27°35'49" E), Muğla city. The area has an average temperature of 19.0°C and an average annual rainfall of 809mm. The samples were collected from a depth of 0 – 30 cm of the rhizospheric soil of *Colchicum balansae* planch plant. The soil sample was fragmented into two fragments, one of which was sterilized at 121 °C for 60 minutes while the other portion wasn't sterilized; the samples were coded according to the bacterial fertilizer treatment they received (Table 1).

Prior to the commencement of experimentation, the physicochemical properties of the collected samples were determined. The soil sample was air-dried and

sieved through a 2 mm sieve. The pH of the soil was determined using pH meter (Jackson, 1958), the organic carbon content of the sample was determined using the Anne method (Schlichting, 1971), Kjeldahl method was employed in determining the total N content of the sample (Bremmer, 1965), the available phosphorus content of the sample was determined according to Olsen method (Olsen, 1954). Moreover, the soil texture was determined according to the description of Bouyoucos (1951), the method reported by Allison & Moodie (1965) was adopted in determining the lime content of the soil, and the field capacity (θ_{fc}) of the soil was determined according to Demiralay (1993).

Table 1. Experimental setup key

Treatment	Definition
K	Unsterilized soil control
Ks	Sterilized soil control
A	Unsterilized soil to which bacterial fertilizer A was applied to.
As	Sterilized soil to which bacterial fertilizer A was applied to
B	Unsterilized soil to which bacterial fertilizer B was applied to
Bs	Sterilized soil to which bacterial fertilizer B was applied to
C	Unsterilized soil to which bacterial fertilizer C was applied to
Cs	Sterilized soil to which bacterial fertilizer C was applied to

Metagenomic Analysis

ZymoBIOMICSTM DNA Miniprep kit (Catalog number: D4300T, D4300 and D4304, Zymo Research/USA) was used to isolate DNA from the soil sample following the manufacturer's description. 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-TACGGCTACCTTGTTACGACTT-3'), 21F (5'-TTCCGGTTGATCCYGCCGGA-3'), 958R (5'-YCCGGCGTTGAMTCCAATT-3'), EukF (5'-AACCTGGTTGATCCTGCCAGT-3'), EukR (5'-TGATCCTTCTGCAGGTTCACCTAC-3') forward and reverse primers were used for amplification of the 16S rRNA gene of Bacteria, Fungi and Archaea respectively. NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) was used to determine the purity and concentration of the isolated DNA.

With the aid of MinION ™ control software, MinKNOW ™ version 0.46.1.9 (R9.4), a sequencing protocol of 48 hours was executed. The read data based on the 1.2.2 rev 1.5 workflows and software Metrichor ™ agent (version 0.16.37960) was obtained. On completing the sequencing, Guppy v3.1.5 software (base-calling and de-multiplexing) was used in converting the sequencing results from fast5 to fastq format. Porechop v0.2.3 software was used in clearing the barcode, and adapter sequences and the universal primers and labels were eliminated by deleting 45 bases from both ends of the sequences. The reads were filtered at 1300 – 1500 bp length.

The cleaned readings were analyzed with a customized workflow using the mothur v.1.39.5 platform. The sequences were purified from chimeric structures by measuring the distances between them with the similarity matrix to be aligned relative to each other; readings showing more than 99% similarity were clustered and formed operational taxonomic units (OTUs). The generated OTUs were compared according to the RDP 16S rRNA database, and their taxonomic annotations were performed. Likewise, the OTUs that were determined as the same genus were associated and statistical results were obtained. Further analyses, such as alpha diversity, beta diversity, and basic coordinate analysis, was performed relative to the organisms with whose OTUs are matched.

Determination of Bacterial count

With the aid of Petroff-hausser counting chamber, the total number of bacteria contained in each of the bacterial fertilizers (A, B, C) were determined. Two mL of the bacterial fertilizer was aseptically dispensed to the soil sample, and an uninoculated sample was set aside to serve as a control.

The bacterial count of the soil sample was determined by introducing 10 g of the soil sample to a flask containing 95 mL of 0.1% (w/v) sodium pyrophosphate solution. The mixture was homogenized for 30 minutes at 100 rpm on an orbital shaker. Afterwards its was serially diluted (10^{-1} to 10^{-6}). To evaluate aerobic mesophilic and spore-forming bacteria, an aliquot from the aforesaid dilution was plated on Tryptone soya agar (TSA, Oxoid, Basingstoke, Hampshire, England), and the plate was incubated at 30 ºC for 2 days. While for the fungal count, an aliquot of the dilution was inoculated on modified Czapek dox agar (Oxoid Code: CM0097) and incubated for 3 days.

Determination of carbon mineralization in soil

At the bottom of a 750 mL glass jar, 80 g of the air-dried soil was moistened up to 80% of its own field capacity. As described by Treuer & Haydel (2011), the moistened soil was inoculated with 2 mL of bacterial fertilizer. A 50 mL beaker containing 10 mL of 1M NaOH was inserted in the middle of the jar containing the moistened soil. The jar was sealed and incubated

at 28 °C. Empty vessels were utilized as blank control. CO_2 gas released due to microbial activity in the soil contained in the jar was held by NaOH.

After every 3 days of incubation, the beaker inserted in the vessel was taken out, and 2 mL of $BaCl_2$ was added to its content till precipitation occurred; 1M HCl was titrated against the mixture with a few drops of phenolphthalein serving as an indicator (Alef & Nannipieri, 1995). In place of the collected beaker, another one containing 1 M NaOH was inserted, the jar was tightly sealed, and the incubation continued.

The carbon generated from microbial activity was expressed in mg per 100 g of dry soil (mg C 100 g^{-1} of dry soil). The ratio of the carbon that undergoes mineralization to the total soil carbon was termed the "Mineralization rate".

Soil carbon mineralization was determined using the relation:
Carbon mineralization % C (CO_2) = ((B – S)×6 P/1)×100
B: the amount of HCl titrated against Blank control, S: the amount of HCl titrated against Sample, P: weight of dried soil sample

Statistical Analyses

Experiments for each treatment were run in triplicate, and the average value is represented. All data obtained from the experiment was statistically analysed using SPSS windows, version 15.0. To determine differences existing among treatments, One-Way Analysis of variance and Tukey HSD multiple comparisons test were conducted.

RESULTS and DISCUSSION

The soil was found to be slightly alkaline, medium light structured, with less $CaCO_3$, less organic matter, very low total nitrogen and medium available phosphorus contents (Table 2).

Table 2. Pysico-chemical properties of the soil sample

Characteristics	Quantity
Sand	68.43 %
Silt	20.24 %
Clay	11.33 %
% C	1.37 ± 0.17
% N	0.04 ± 0.01
C/N	34.67 ± 2.91
Available P	6.04 ± 0.48
$CaCO_3$	0.19 ± 0.10
pH	7.63 ± 0.04
Field capacity	18.57 %

The soil sample was found to exhibit aerobic mesophilic bacterial count of 1.3 x 10^4 cfu mL^{-1}, spore forming bacterial count of 2 x 10^4 cfu mL^{-1} and fungal count of 4.3 x 10^4 cfu mL^{-1} (Table 3).

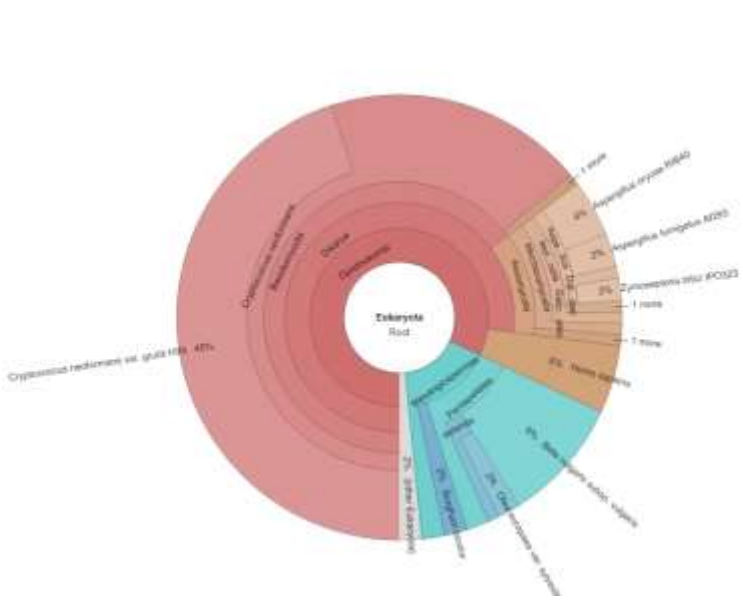
Table 3. Bacterial count enumeration

Sample	*Description*
Soil	1.3 x 10^4 cfu mL^{-1} aerobic mesophilic bacteria, 2 x 10^4 cfu mL^{-1} spore forming bacteria, 4.3 x 10^4 cfu mL^{-1} fungi, yeast and mould count.
Fertilizer A	*Bacillus pumilus, B. amyloliquefaciens, B. megaterium, B. subtilis, B. licheniformis, Trichoderma harzianum and T. konigii*. Total microbial count: 1.2 x 10^8 cfu mL^{-1}.
Fertilizer B	*Azorhizobium, Azotobacter and Azospirillum*: 3.1 x 10^8 cfu mL^{-1}.
Fertilizer C	*Azotobacter* spp with *Bacillus licheniformis* (3 x 10^3 cfu mL-1), *B. subtilis* (2 x 10^3 cfu mL^{-1}), *B. megatarium* (1 x 10^3 cfu mL^{-1}), *Pseudomonas putida* (8 x 10^6 cfu mL^{-1}), Total aerobic mesophilic bacterial count (1.3 x 10^{10} cfu mL^{-1})

cfu mL^{-1}: Colony forming units per millilitre

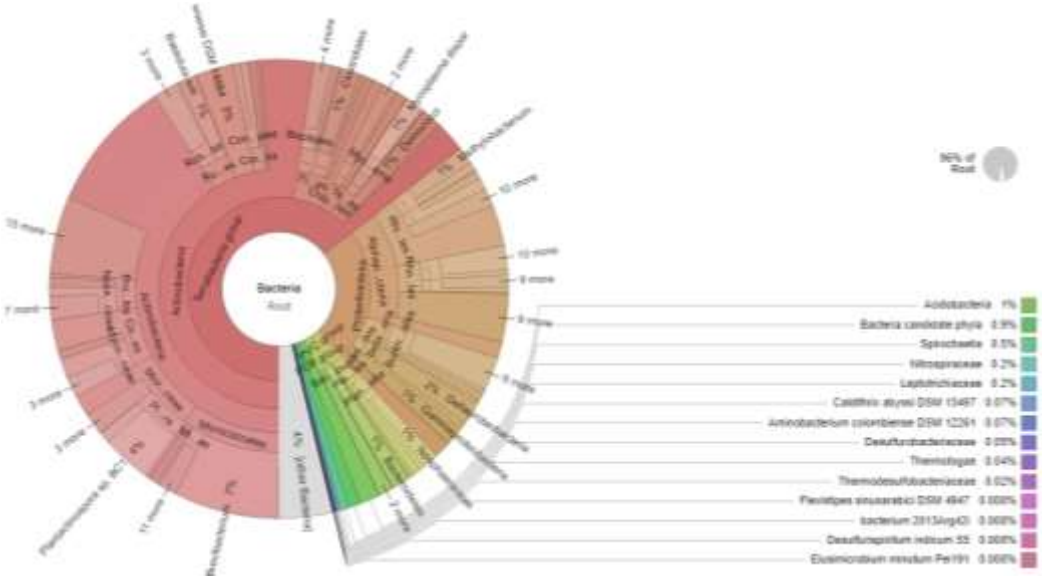
As a result of metagenome analysis, *Cryptococcus neoformans var grubii* H99 was found to be the dominant eukaryotic organism in the soil sample analyzed (45%); other prominent organisms are *Aspergillus oryzae* RIB40 (4%), *Aspergillus fumigatus* AF293 (2%) and *Zymoseptoria tritici* IPO323 (2%). Eukaryotic microorganisms have the ability to degrade polysaccharides that cannot be biologically degraded into smaller components by the exoenzymes of the bacterial domain, on the archaea domain, *Halopropundus* sp. MHRI and *Thermococcus* were found to both be 7%, while in the domain bacteria, *Brevibacterium* was observed to be 7%, thereby making it the most abundant in comparison to other bacterial genera (Figure 1). On the phylum level, **Actinobacteria>Proteobacteria>Chlorofleksi>Acidobacteria>Gemmatimonadetes>Planctomycetes** was found to be approximately 96% dominant in the soil sample. Actinobacteria is of immense importance in agriculture and forestry.

In soil, they act more like fungi, decomposing organic matter, thereby making their nutrients readily available for plant utilization. On the genus level, **Micromonospora> Blastococcus> Solirubrobacter> Rubrobacter>Mycobacterium> Microvirga** was found to be approximately 17% thereby becoming the dominant genus. On the species level, *Conexibacter woesei* DSM 14684 (3%), *Plantactinospira* sp. BC1 (3%), *Solibacter usitatus* (1%), *Mycoplasma dispar* (1%), *Baekduia soli* (1%) were found to be the dominant species in the soil.



a. Domain Eukaryota

b. Domain Archeae



c. Domain Bacteria

Figure 1. Composition and % abundance of a. Eukaryota b. Archaea c. Bacteria in the analyzed soil sample

The daily graph gave more meaningful results in terms of determining the presence of the interactions between the indigenous bacteria in the soil and the bacteria introduced to the soil through the application of the bacterial fertilizer. The highest carbon mineralization was observed to have occurred on the 3rd day, with the exception of the unsterilized soil treated with bacterial fertilizer C (Figure 2). This can be attributed to the utilization of easily decomposed carbon sources by microorganisms. The fluctuation in daily carbon mineralization was striking for the type of soil organic matter and the capacity of the microbes to decompose organic matter.

An adaptation phase was observed in the cumulative curve graph for the first 9 days, followed by a gradual increase. The cumulative carbon mineralization was the lowest in the sterilized control soil and the highest in unsterilized soil treated with bacterial fertilizer C, followed by sterilized soil onto which bacterial fertilizer C was applied. Cumulative mineralized carbon increased with incubation time, and its increase gradually slowed down (Figure 3).

In sterilized soil treatment, the highest carbon mineralization was observed; this might be attributed to the interaction between the introduced bacteria contained in the bacterial fertilizer and the resident microbes of the unsterilized soil.

On applying the bacterial fertilizer A, B and C separately to the sterilized control soil, whose initial mineralization rate was 1.1%, mineralization rates of

5.12%, 3.54% and 10.78% were recorded, this shows that the application of the bacterial fertilizer to the soil increased the rate of carbon mineralization of the sterilized control soil by 365.45%, 221.82% and 880% respectively. So also, on inoculating the unsterilized control soil - whose initial carbon mineralization rate was 5.61 - with bacterial fertilizers A, B and C, carbon mineralization rate of 7.03%, 6.15% and 12.95% were recorded, this signifies that application of the aforementioned bacterial fertilizers increased the rate of carbon mineralization of the soil by 25.31%, 9.63% and 130.84% respectively.

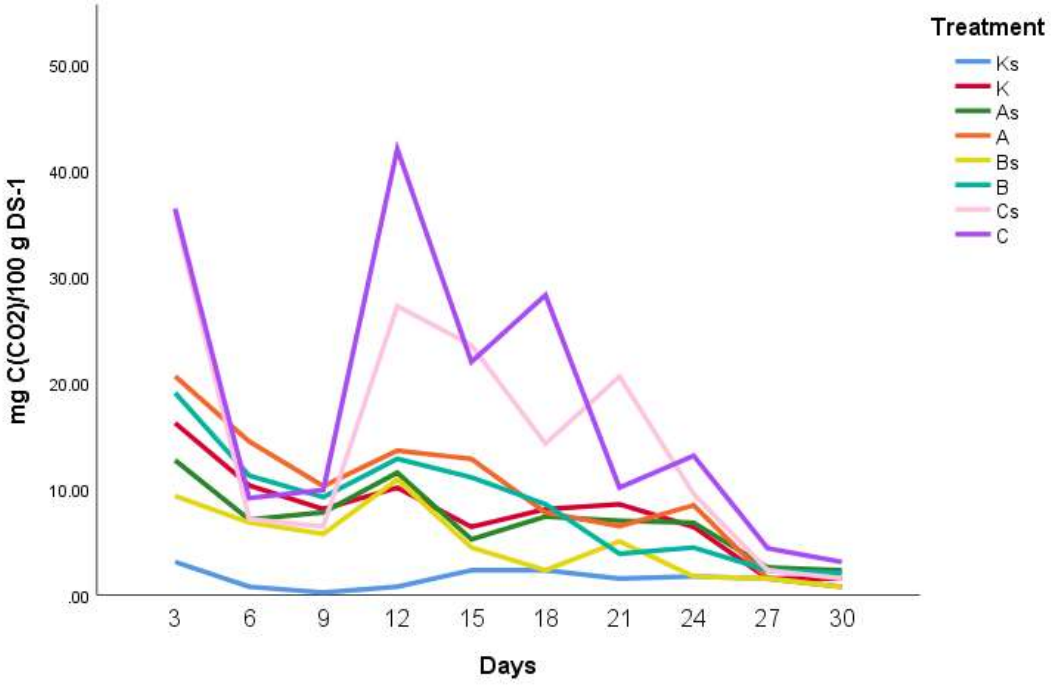


Figure 2. Daily carbon mineralization of soils, mg C (CO_2) 100 g^{-1} Dry Soil

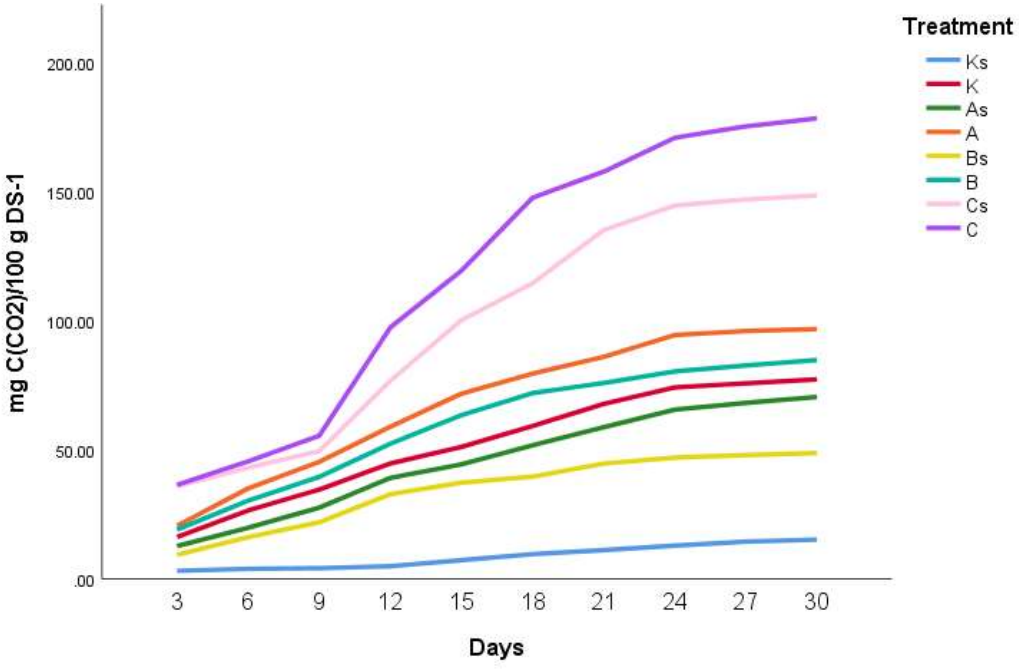


Figure 3. Cumulative carbon mineralization of the analyzed soils

Unsterilized soil treated with bacterial fertilizer C shows the highest carbon mineralization rate of 12.95%, (Figure 4) while the lowest rate (1.1%) was shown by the sterilized control soil. In ecologically balanced soils, the carbon mineralization rate is usually around 10%. Higher value signifies the release of more carbon into the atmosphere, and this has a detrimental effect to the ecosystem.

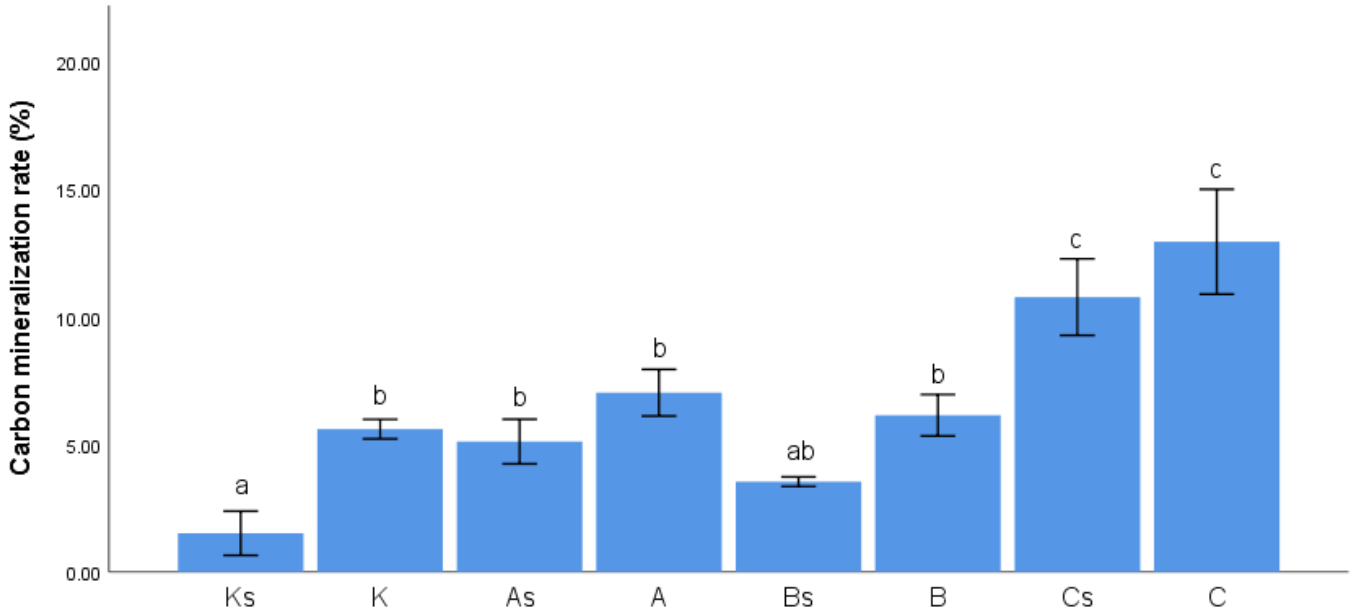


Figure 4. Carbon mineralization rates of the analysed soils (%). Each value represents the mean of three replicates ± SE. Different letters indicate means that are significantly different from each other (ANOVA: Tukey test, $p < 0.05$)

On analyzing the carbon mineralization rate of the treatments using one way analysis of variance, it was found that significant differences exist among the two sets of treatments (i.e., the sterilized soil sample set and the unsterilized soil sample set) (Table 4).

Table 4. Carbon mineralization in soil samples treated with the three bacterial fertilizers

Soil	Carbon mineralization (C mg 100g^{-1} soil)
K	75.33 ± 2.25
Ks	21.11 ± 3.94^{a3}
A	96.87 ± 1.07^{b2}
As	73.40 ± 1.21^{a2c3}
B	85.98 ± 2.50
Bs	48.20 ± 1.75^{a3c3}
C	173.28 ± 3.67^{b3}
Cs	142.43 ± 6.98^{a3c3}

Same alphabet signifies the existence of significant difference between treatments $a_1b_1c_1 \leq 0.05$, $a_2b_2c_2 \leq 0.01$, $a_3b_3c_3 \leq 0.001$

While working to determine C mineralization model in garden soil treated with both inorganic and microbial fertilizer, Sarkar & Rakshit (2020) reported a significant increase in cumulative carbon dioxide flows of 137.25 mg CO_2 100 g^{-1} soil in the treated soil in comparison to 46.20 mg CO_2 100 g^{-1} soil recorded in the control soil. They also recorded a daily carbon mineralization of 0.26 and 1.78 mg C 100 g^{-1} soil in the control soil and 0.30 and 3.23 mg C 100 g^{-1} soil day^{-1} in the treated soil and concluded that their application of the fertilizer to the soil increased carbon mineralization rate. Their findings corroborate that of this research. So also, Salehi et al. (2017), while investigating the effect of organic and inorganic fertilizer application on soil CO_2 flux, came to the conclusion that the application of the said fertilizers significantly increases soil CO_2 flux. This study differs from other studies because instead of utilizing both organic and biofertilizer, only biofertilizer was utilized.

Among the three bacterial fertilizers used in the study, we found a low carbon mineralization rate in soil treated with bacterial fertilizer containing rhizobacteria. While the highest increase in carbon mineralization was recorded in soil treated with the fertilizer containing *Azotobacter* spp. and other plant growth-promoting bacteria in equal proportion.

CONCLUSION and RECOMMENDATIONS

This study confirms that bacterial fertilizer application enhances soil carbon mineralization rate. Though the application of bacterial fertilizer to agricultural soil has a positive effect on crop yield, it has a detrimental effect ecologically because it increases the emission of CO_2 into the atmosphere. A viable approach to compensating for the increased efflux of CO_2 from soils into the atmosphere is carbon sequestration, and one of the most effective and promising strategies of achieving carbon sequestration is the utilization of biochar. Future research should try to mitigate the issue of CO_2 emission into the atmosphere through the utilization of novel and cost-effective strategies.

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Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

Conflict of Interest Statement

The authours report no conflict of interest.

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