Humic Acid Improves Greenhouse Tomato Quality and Bacterial Richness in Rhizosphere Soil

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ABSTRACT: Humic acid (HA) has attracted increasing attention as a new type of organic fertilizer in horticultural production, such as greenhouse-planted cherry tomato. However, we need more information to evaluate the effects of HA on soil rhizosphere bacteria and tomato performance under greenhouse conditions. In this study, greenhouse-planted cherry tomato was observed with HA added at dosages of 1500, 3000, 4500, and 6000 kg ha$^{-1}$, respectively. The other two organic fertilizers [farmyard manure (FM) and commercial organic fertilizer (COF)], were used as comparison with a dosage of 3000 kg ha$^{-1}$. Illumina MiSeq sequencing was conducted for bacterial diversity analysis, and tomato quality analysis based on total soluble solids, titratable acid, and sugar–acid ratio was performed for different fertilizer treatments. The results revealed that HA application resulted in the best flavor, compared to CK without the organic fertilizer used and with the other two organic fertilizers. The Chaol estimator and Shannon index showed that fertilizer addition decreased microbial diversity but increased species richness. At a dosage of 3000 kg ha$^{-1}$, the effects of different fertilizers were ranked as HA > FM > COF. Our findings offered suggestions to reasonably optimize cherry tomato organic fertilizer application.

1. INTRODUCTION

Tomato (Lycopersicon esculentum Mill.) is one of the most important economic crops worldwide. In 2019, China as the largest tomato-producing country in the world has a tomato planting area of 5.05 million ha and harvests 29.93 million tons annually. Zhejiang province, located in southeast China (118°01′–123°08′E, 27°01′–31°10′N), is one of the most important vegetable production areas in China. Tomato has a well-developed root system, and rhizosphere is a key interface for healthy growth and soil-borne disease prevention. Greenhouse planting is the main cultivation pattern of tomato, and it is characterized by high cost, high productivity, and high profits. Application of new and suitable fertilization are quite important to achieve high yield and quality in greenhouse-planted tomato and to avoid soil salinity caused by the chemical fertilizer.

Due to the adverse effects of chemical fertilization on greenhouse soils, nowadays, special attention has been paid to organic and biological fertilizers or those technical manage-
affecting the plant metabolism and thus influence the structure of the rhizosphere microbial community.\textsuperscript{15}

The rhizosphere is an important interface for the interactions between tomato roots, soils, and microorganisms and exhibits intensive element and energy exchange, and HA application can promote the bacterial community and affects plant growth, health, and quality.\textsuperscript{16–18} Consequently, HA application can influence the information communicated and energy transferred between the plants and the soil within the rhizosphere soil environment, reduce the diseases and stresses, and increase the crop yield.\textsuperscript{16} Changes in the soil environment and plant rhizosphere microbial community lead to changes in the quality of produce, such as pear, maize, and rice.\textsuperscript{19–21} Previous studies based on rhizosphere metabolomics have focused on metabolites and plant health.\textsuperscript{22–24} As discussed, HA impacts tomato rhizosphere bacteria, and thus, it is reasonable to propose that the addition of HA may affect tomato quality.

This article hypothesized that the application of HA and other organic fertilizers would alter greenhouse tomato rhizosphere bacterial diversity and improve tomato quality. The aims of this study were to (1) explore the correlations between tomato quality and different fertilizer treatments; (2) reveal the relationships among HA application and tomato rhizosphere bacterial diversity; and (3) determine the optimal dosage of HA in greenhouse-planted cherry tomato cultivation.

2. RESULTS AND DISCUSSION

2.1. Root Characteristics. The effect of treatments HA, farmyard manure (FM), commercial organic fertilizer (COF), and control check (CK) on cherry tomato root characteristics are shown in Figure 1. Compared to CK, HA treatment markedly increased the number of fibrous tomato roots, and the root tips and root forks increased at the same time. COF and FM treatments increased the number of fibrous tomato roots at the same time. These results indicated that tomato root growth was significantly enhanced through organic fertilizer addition, and HA was the best.

2.2. Effects of Fertilizers on Tomato Quality. The total soluble solids (TSS), titratable acid (TA), and sugar–acid ratio (SAR) of cherry tomato are shown in Table 1. Tomato quality factors were significantly higher in all fertilization treatments than in the CK treatment ($P < 0.05$). TSS increased by 2.00\% in the S1 treatment, 2.78\% in the S2 treatment, 2.89\% in the S3 treatment, 5.34\% in the S4 treatment, 0.89\% in the S5 treatment, and 1.34\% in the S6 treatment. In contrast, TA increased by 1.00\% in the S1 treatment and decreased by 6.01\% in the S2 treatment, 9.52\% in the S3 treatment, 11.7\% in the S4 treatment, 0.70\% in the S5 treatment, and 3.51\% in the S6 treatment.

In this experiment, cherry tomato was used as the trial crop. According to former studies, the higher the SAR value, the better the flavor. Table 1 shows that tomato quality varies under different fertilizer applications. For the S1, S2, S3, and S4 treatments, as the HA application dosage increased, tomato TSS increased. The TSS values with HA application were higher than that with FM and COF applications. As far as the reason concerned, nutrition content increased as the HA dosage increased, increasing TSS at the same time. In contrast, the TA value decreased as the HA dosage increased, and thus, the SAR value increased. For TSS and SAR, the values of the FM and COF (S5 and S6, respectively) treatments were below the values of S1. From Table 1, it could be concluded that tomato TSS and SAR values could be ranked in order as HA treatment > FM treatment > COF treatment. Compared with that in the FM and COF treatments, the tomato flavor in the HA treatments was better.

2.3. Changes in Rhizosphere Bacteria Diversity in Different Fertilizer Treatments. Sequence readings and operational taxonomic units (OTUs) were obtained from rhizosphere soil bacteria from all 20 samples (Table 2). The results were grouped at the 97\% similarity level, and the OTUs

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Root characteristics under different treatments (CK stands for control check, HA stands for humic acid treatment, COF stands for commercial organic fertilizer treatment, and FM stands for farmyard manure treatment).

Table 1. Tomato Quality in Terms of TSS, TA, and SAR under Different Fertilization Treatments\textsuperscript{a,b,c}

| treatment | TSS, % | TA, % | SAR |
|-----------|-------|-------|-----|
| S1        | 9.16 ± 0.351bc | 0.403 ± 0.0006a | 22.7 ± 2.36 |
| S2        | 9.23 ± 0.085b | 0.375 ± 0.0006d | 24.6 ± 2.45 |
| S3        | 9.24 ± 0.010b | 0.361 ± 0.0045e | 25.6 ± 2.36 |
| S4        | 9.46 ± 0.065a | 0.352 ± 0.0040f | 26.9 ± 2.78 |
| S5        | 9.06 ± 0.015de | 0.392 ± 0.0035b | 23.1 ± 2.56 |
| S6        | 9.10 ± 0.050cd | 0.385 ± 0.0055c | 23.6 ± 2.47 |
| S7        | 8.98 ± 0.025e | 0.399 ± 0.003a | 22.5 ± 2.15 |

\textsuperscript{aDifferent lowercase letters indicate a significant difference at $P < 0.05$. TSS, total soluble solids; TA, titratable acid; and SAR, sugar–acid ratio.}

Table 2. Sequence Readings of Raw Tags, Valid Tags, Effectiveness, and Number of OTUs of Rhizosphere Soil Bacteria under Different Fertilization Treatments\textsuperscript{a}

| treatment | raw tags | valid tags | effectiveness/% | number of OTUs |
|-----------|----------|------------|-----------------|----------------|
| TEST1 or S1 | 54,480.67 | 51,774.00 | 95.03 | 2183 |
| TEST2 or S2 | 55,482.00 | 50,933.00 | 91.80 | 2550 |
| TEST3 or S3 | 53,429.00 | 50,036.67 | 93.65 | 2392 |
| TEST4 or S4 | 55,300.33 | 51,450.33 | 93.04 | 2466 |
| TEST5 or S5 | 52,867.00 | 50,377.67 | 95.29 | 2009 |
| TEST6 or S6 | 55,675.33 | 52,977.33 | 95.15 | 2183 |
| CK or S7 | 47,969.00 | 42,940.00 | 89.52 | 2085 |

\textsuperscript{aEffectiveness is the ratio of valid tags to raw tags.}
were clustered. Quality control was conducted using the MiSeq PE300 platform for all sequences, and the raw tags ranged from 47,969.00 to 55,675.33, the valid tags ranged from 42,940.00 to 52,977.33, the effective ratio ranged from 91.80 to 95.29%, and the OTU number ranged from 2009 to 2550.

Based on the number of unique OTUs observed in each soil sample, the Chao1 estimator and Shannon index were used to determine the abundance and diversity of the rhizosphere soil bacteria. The Shannon index reflects species abundance and evenness, while the Chao1 index reflects species richness. For S1 to S4, the OTUs increased compared to CK by 4.7, 22.3, 14.7, and 18.2%, respectively. In S5, with the FM, OTUs decreased by 3.645% compared to that with CK, which may have been related to the fact that FM contained antibiotics that could have reduced the bacterial abundance. In S6, with COF, OTUs increased by 4.700% compared to that with CK.

As shown in Figure 2, compared to CK, the Chao1 estimator increased and the Shannon index decreased with fertilizer treatment. Treatments S1 to S4, with HA, yielded higher values than organic fertilizer treatment. Regarding the Chao1 estimator, the values for S1, S2, S3, and S4 were 3320, 3540, 3308, and 3490, respectively, while the Chao1 estimator values were 2930 for FM and 3210 for COF. For the Shannon index, the values for S1, S2, S3, and S4 were 9.13, 9.32, 9.20, and 9.16, respectively, while the Shannon index values were 8.68 for FM and 9.18 for COF. In general, fertilizer addition decreased microbial diversity but increased species richness. The Chao1 estimator and Shannon index of the COF treatment in S6 were higher than those of the FM treatment in S5.

A rarefaction curve and the Shannon–Wiener index (Figure 3) of all soil samples were obtained for different fertilization treatments, and the results showed that all curves sharply
increased within a sequencing depth of approximately 5000, after which the curves reached their asymptotes. This indicated that the data generated in this study were sufficient for the analysis of the diversity of rhizosphere soil bacteria in greenhouse-planted tomato.

2.4. Effects of Fertilizers on Tomato Rhizosphere Bacterial Taxa. At the phylum level, the bacterial communities of the tomato rhizosphere soil under different treatments are shown in Figure 4. The results revealed that with different fertilizer treatments, the bacterial richness was equivalent to the number of OTUs determined at the 97% similarity level, and the relative abundance was as follows: Proteobacteria was the most abundant bacterial phylum and accounted for 19.1–32.2%, Actinobacteria accounted for 14.9–24.4%, Chloroflexi accounted for 11.3–32.2%, Acidobacteria accounted for 9.59–18.4%, Firmicutes accounted for 3.97–12.67%, and Gemmatimonadetes accounted for 4.58–9.54%. Compared to that in S7, in the CK experiment, when HA and FM were applied, the abundance of Proteobacteria decreased. Moreover, at the largest HA dosage (6000 kg·ha⁻¹), Proteobacteria decreased the most. Chloroflexi abundance increased obviously with fertilizer application, and the proportion increased more with FM and COF application than with HA application.

At the class level, the bacterial community of the tomato rhizosphere soil under different cropping patterns is shown in Figure 5. The results revealed that at the class level, the main bacterial communities were Thermoleophilia, Ktedonobacteria, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Gemmatimonadetes, Bacilli, Acidobacteria, Betaproteobacteria, Solibacteres, and Thermomicrobia. Compared with CK (S7), the Ktedonobacteria abundance increased the most. The difference was obvious in Ktedonobacteria, Alphaproteobacteria, and Actinobacteria under HA, FM, and COF application.

Principal coordinate analysis (PCoA) statistical methodologies were used to identify the bacterial communities among the samples. The results are shown in Figure 6. The results revealed that the first two principal components (PC1 and PC2) explained 43.22 and 13.45% of the variability for the tomato rhizosphere soil bacterial community, respectively. There were three clusters: CK application (S7), HA application (S1, S2, S3, and S4), and organic fertilizer.
application (S5 and S5). The observed difference in the tomato rhizosphere soil under different fertilizer applications and their high similarity suggested different and rapidly changing bacterial communities throughout the experiment during greenhouse tomato planting.

Bacteria play an irreplaceable role in maintaining the balance of soil ecosystems, forming crop rhizosphere microbial ecosystems, and affecting nutrition transportation and transformation in soil. Bacteria, therefore, affect the growth of crops and the accumulation of nutrient elements. Microorganisms dynamic including bacteria was affected by plant root exudates and the associated changes in rhizosphere soil. Different fertilizer types and application methods may affect bacterial diversity, increasing or decreasing the number or structure of certain microorganisms, thus affecting soil bacteria ecological systems. In the present study, the soil microorganisms of greenhouse-planted tomato were studied, and a comparison of tomato quality, bacteria diversity, and functional potentials was carried out under HA, FM, and COF application with different dosages. Tomato quality analysis revealed that the tomato TSS content increased with the HA application dosage, and among the three fertilizers, HA application had the best effect on increasing the tomato quality and had an obvious effect on tomato quality improvement. HA, as a new organic fertilizer, could have an obvious effect on tomato quality improvement. HA also improved the crop growth status and increased nutrient accumulation, which was in accordance with the findings of former studies.

Soil bacteria diversity analysis revealed that for the Chaol estimator, the values under HA application were higher than those under FM and COF application, and the values of all fertilizer treatments were lower than those under CK conditions. Studies have shown that HA application stimulates photosynthesis, accelerates the biomass accumulation rate of plants, and leads to an increased bacterial abundance and that increasing the soil active microbial biomass and short-term fertilizer input increases bacterial richness but decreases bacterial diversity in the rhizosphere. Organic material in soil, which is mainly composed of the byproducts of bacterial decomposition, can provide nutrient elements for soil bacterial growth; this is conducive to microbial reproduction and increasing quantity. The investigation of the tomato rhizosphere soil under different cropping patterns at the phylum level revealed that Proteobacteria and Chloroflexi changed obviously with the application of different fertilizers. The phylum Proteobacteria contains a variety of bacteria that can fix nitrogen and promote soil nutrient cycling, including carbon and sulfur circulation. A previous study found that Proteobacteria was significantly positively correlated with soil organic matter content. The phylum Chloroflexi is composed of a diverse group of organisms that include anoxygenic photoautotrophs, aerobic chemoheterotrophs, thermophilic organisms, and anaerobic organisms that can obtain energy by the reductive dehalogenation of organic chlorinated compounds. Chloroflexi species have important functions in bacterial diversity function building and maintenance, and the increase in Chloroflexi indicated an increase in the ability of the soil to inhibit or reduce the growth of harmful microorganisms and disease transmission, thereby improving the comprehensive disease resistance of the soil. Maintaining a reasonable level of Chloroflexi species is important for healthy soil cultivation. Short-term fertilizer addition decreased microbial diversity but increased species richness, which was inconsistent with former studies.
4. MATERIALS AND METHODS

4.1. Field Trial Design. The field experiment was conducted in the greenhouse trial field at Zhejiang Academy of Agricultural Sciences, located in the city of Shaoxing (30°04′N, 120°64′E), Zhejiang Province, China, from September 2020 to May 2021. The trial soil was classified as yellow-brown soil, and its characteristics were as follows: pH 6.64, 20.6 g·kg⁻¹ organic material content, 1.35 g·kg⁻¹ total N content, 0.65 g·kg⁻¹ total K content (K₂O), and 0.45 g·kg⁻¹ total P content (P₂O₅).

Tomato seeds were sown in September 2020 in trays and transplanted when three–four leaves were grown from the plan. Field trials were conducted under greenhouse conditions in separate plots, each measuring 60 m², with three replicates. The plots were separated by blank experimental plots 1 m in width. Planting was carried out according to the local cultivation practices, with a border width of 0.9 m, a ditch depth of 0.25 m, a continuous furrow border width of 1.6 m, and two rows planted every border. Young tomato plants were transplanted in January 2021 and planted in a triangle planting arrangement. The row spacing was 40 cm, and 135 tomatoes were transplanted in each plot. The trial plots were divided into six types, S1–S6, with the control (CK) plot type designated S7. HA and two other widely used organic fertilizers were applied to greenhouse-planted cherry tomato.

Plot types S1, S2, S3, and S4 were treated with different dosages of HA with three replicates each, plot type S5 was treated with FM, with three replicates, plot type S6 was treated with the COF, with three replicates, and two blank plots (S7) were not treated with the fertilizer. There were 20 trial plots in total. The fertilizers applied in each trial are shown in Table 3. All the fertilizers were applied when the tomato plants were transplanted.

4.2. Materials and Cherry Tomato Breeding. Seeds of the "Jinhu" cherry tomato variety were supplied by Zhejiang Academy of Agricultural Sciences. HA was supplied by Zhejiang Fengyu Ecological Technology Co., Ltd., Jinhua, Zhejiang Province, China. The HA was supplied by a local chicken farm, and the characteristics were as follows: pH 7.5, 35% HA, 45% organic material, and 5.05% (dry weight) total nutrient content (N + P₂O₅ + K₂O). FM was supplied by a local chicken farm, and the characteristics were as follows: pH 7.5, 18.7% crude protein, 2.5% fat, 13% ash, 11% carbohydrates, 7% fiber, and 5.09% total nutrient content (N + P₂O₅ + K₂O). COF was supplied by Shaoxing Agricultural Production Material Co., Ltd., Shaoxing, Zhejiang Province, China. The characteristics were as follows: pH 7.5, 35% organic material, and 5% total nutrient content (N + P₂O₅ + K₂O) by dry weight.

4.3. Sampling of Cherry Tomato and Rhizosphere Soil. Samples were collected in May 2021 during the peak harvest season, when soil microorganisms were very active. The tomato rhizosphere soil was collected using the five-point method. The diagonal method was used to select five points in each trial plot. Surface plants and mulch were removed before sampling, and the rhizosphere soil with 2 cm of the tomato roots was collected using a shovel. Soil samples from different points in the same plot were mixed and sieved through a 2 mm mesh. All samples were placed in plastic bags, transported to the laboratory on the day of sampling, and stored at a temperature of −20 °C for high-throughput sequencing according to previous reports.

Tomato samples were collected on May 1, 2020. Ripe tomatoes weighing approximately 200 g from different points in the plots and heights on the plants were collected for each sample. All samples were placed in plastic bags and transported to the laboratory on the day of sampling for quality analysis.

4.4. Soil DNA Extraction and Quantitative Polymerase Chain Reaction Analysis. Three samples each were collected from trials S1 to S6 and two samples were collected for S7 for a total of 20 samples. Soil deoxyribonucleic acid (DNA) was extracted from soil samples (0.5 g wet weight) using an E.Z.N.A Soil DNA Kit (Omega, USA) according to the manufacturer's instructions. The extracted DNA was diluted in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C until use.

In this study, four rounds of real-time polymerase chain reaction (PCR) were conducted to determine the abundances of different taxonomic levels of bacteria using different specific primer sets. Microbial community genomic DNA was extracted from soil samples using the E.Z.N.A soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable V3–V4 region of the bacterial 16S rRNA genes was amplified with the primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GACTACHVGGGTWTCTAAT-3′) using an ABI GeneAmp9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA genes was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min.
at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a single extension at 72 °C for 10 min, and ending at 4 °C. The PCR mixtures contained 4 μL of 5X TransStart FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of the forward primer (5 μM), 0.8 μL of the reverse primer (5 μM), 0.4 μL of TransStart FastPfu DNA polymerase, and 10 ng of template DNA, and H₂O was added to bring the final volume up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel, purified using the Agencourt AMPure XP DNA gel extraction kit (Agencourt Biosciences, Union City, CA, USA) according to the manufacturer’s instructions and quantified using the Quantus fluorometer (Promega, USA).45–47

4.5. Illumina MiSeq Sequencing and Diversity Analysis. Purified amplicons were pooled in equimolar ratios and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio BioPharm Technology Co. Ltd. (Shanghai, China).

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp version 0.20.0, and merged using FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded. (ii) Only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was 0.1. Reads that could not be assembled were discarded. (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, with exact barcode matching and two-nucleotide mismatch in primer matching.46,47

OTUs with a 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed using RDP Classifier version 2.2 against the 16S rRNA database (e.g., Silva v138) using a confidence threshold of 0.7.46,50–52

4.6. Tomato Quality Determination. Tomato root was selected from three to five samples, and the root length, root tips, and number of root forks were recorded. During the full fruiting period, additional samples of the mature tomato fruit were collected from five individual plants to determine the tomato quality. According to former studies, the TSSs, TA, and SAR are key indicators of tomato quality.3 TSS was measured using the refractometer method, TA was titrated with 0.1 mol/L of NaOH, and the SAR was defined as the ratio of TSS to TA. For each treatment, five parallel samples were examined, and the average values of TSS, TA, and SAR were analyzed.53–55

4.7. Statistical Analysis. Statistical analyses were performed similarly to previous reports.56,57 SPSS 17.0 was used to investigate the correlations between tomato quality and the TSS, TA, and SAR. The histogram was visualized using Origin 2021.
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