Effects of microbial fertilizer on apple fruit quality

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Abstract: Pre-harvest factors have a great impact on fruit quality. In order to study the effects of pre-harvest microbial fertilizer on fruit quality, we applied Pseudomonas fluorescens and Bacillus amyloliquefaciens to ‘Starkrimson’ apples during maturation. We found that microbial fertilizer treatment can significantly increase the content of fruit aroma and soluble solids, single fruit weight and brittleness, reduce the organic acid content of the fruit during maturation. These findings provide reference for the scientific selection of fertilizer.

1. Introduction
Apple is a major source of phytochemicals such as flavonoids and phenolic acids, which have been shown to play important roles in the antioxidant defense of human tissues[1].

Many factors affect fruit quality, including pre-harvest fertilization. Although fertilization is an effective strategy, overuse can cause significant environmental damage. It is therefore important to develop new strategies to confer increased quality without the drawbacks of traditional fertilizer[2]. Rhizosphere-promoting bacteria (PGPR) are rhizosphere microorganisms that can directly or indirectly promote plant growth, increase crop yield, and control pests and diseases. Pseudomonas fluorescens and Bacillus amyloliquefaciens are comparatively newer PGPRs, and the information on the role of these organisms in growth promotion is sparse [3]. For the first time, Pseudomonas fluorescens and Bacillus amyloliquefaciens were selected in this experiment to study the effects of pre-harvest application on the fruit quality.

2. Materials and methods

2.1 Materials
Five-year-old ‘Starkrimson’ apples were sampled from Jinling Town, Zhaoyuan City, Shandong Province. For each treatment, 60 apples with uniform size, no signs of pest or disease, and no mechanical damage, were picked from the same height of the canopy from 20 trees. The treatment group was treated with fertilizer on September 10, 2018. Single fruit weight, soluble solids, fruit hardness, brittleness, fruit cells, volatile matter and related genes are measured when the fruit is ripe.

The microbial fertilizer used in the experiment was a mixture of Pseudomonas fluorescens and Bacillus amyloliquefaciens, in the microbial fertilizer group, each tree was treated by dissolving 5 g of Pseudomonas fluorescens (500 million CFU per gram) and 5 g of Bacillus amyloliquefaciens (20 billion CFU per gram) in 5 L of tap water, followed by thorough mixing. The resulting mixture was applied evenly around the base of the trees, while tap water alone was applied to the control group.
2.2 Methods

2.2.1 Determination of Single Fruit Weight, Soluble Solids, Fruit Hardness, Fruit Brittleness

Six fruits were randomly collected for each treatment, and the weight of a single fruit was determined using a balance. The PAL-1 digital saccharimeter was used for the measurement. After beating, the homogenate was filtered with four layers of gauze, and 0.2 mL of the filtrate was directly measured. Then, the apples were treated six times for each treatment and averaged.

Fruit hardness and brittleness were measured using a TA. XT plus type texture analyzer (Stable MicroSystems, UK, P/2 column probe with a diameter of 2 mm). The pre-measurement and measurement speeds were set as 2 and 1 mm·s⁻¹, the measurement speed was 5 mm·s⁻¹, the penetration depth was 10 mm, and the minimum sensing force was 10 g. The data were automatically analyzed and calculated using the Texture Exponent 32 software[4].

2.2.2 Determination of Volatile Substances and Related Genes

Extraction was carried out using a solid phase micro-extractor (Supelco, USA), which was equipped with a SPME fiber extraction head of 50/30 μm DVB/CAR/PDMS. After washing and chopping the fruit, 20 g of samples were accurately weighed into a 50-mL Erlenmeyer flask. Then, 5 μL of internal standard 3-nonanone (0.1 mg·mL⁻¹) was added. After capping and sealing, the mixture was equilibrated for 10 minutes, and inserted into a pre-aged fiber extraction head for 35 minutes for GC-MS detection. Quantitative method: 5 μL of 3-anthrone with a concentration of 0.1 mg·mL⁻¹ was selected as the internal standard for the selective ion detection (SIM), in order to quantitatively analyze the component. The calculation formula was as follows: content of each component of the aroma substance (μg g⁻¹) = [peak area of each component / peak area of internal standard × internal standard concentration (mg·mL⁻¹) × 1,000] / sample amount (g)[4].

The method reported by Gwanpua et al. was used with slight changes[5]. Total RNA was extracted using the rapid universal plant RNA extraction kit (Beijing Huayueyang Biotechnology). We set the expression levels of AAT1, AAT2, LOX, ADH and HPL as 1, and calculated the relative expression levels of treatment.

Table 1. Primers for the qRT-PCR amplification of aroma substance-related gene expression

| Gene name | Primer sequence(5′-3′) |
|-----------|------------------------|
| AAT1      | F: GCTGGATCTGCTTTGTTC R: TGGTTACTGGATGCGTAT |
| AAT2      | F: GGATTACTCAAGGAACTAA R: GACACAACTCATACATTGC |
| LOX       | F: GATGGTCTCCTCTGATGG R: CTTCGTGTCCCTTATTTTG |
| ADH       | F: CCACCAACAAGCAATGAA R: ACCAACACTCTCCACAT |
| HPL       | F: TAGGAGGGAAATGAGAGG R: AGAGAACAAGCAGGAGGT |
| Actin     | F: TGAACGAATGAGCAAGAATTACT R: TACTCAGCTTTGGCAATCCACAT |

2.2.3 Statistical Analysis

All experiments were performed in triplicate and were reported as the mean ± standard deviation (SD). The data were analyzed by one-way ANOVA using the SPSS 20.0 package followed by Duncan’s multiple e-range tests to determine significant differences. Means were considered statistically significantly different at P< 0.05.

3. Results

3.1 Effects of Microbial Fertilizer on the Fruit Quality

3.1.1 Single Fruit Weight, Soluble Solids, Fruit Hardness, Fruit Brittleness

The fruit weight, content of soluble solids, and fruit brittleness of the treatment group were higher than...
those of the control group (Table 2), which were 7.54%, 7.38%, and 26.32% higher than the control group, respectively, and the fruit hardness was 2.0% lower than that of the control group.

Table 2. Differences in the levels of single fruit weight, content of soluble solids, fruit hardness, fruit brittleness.

|                      | Single fruit weight (g) | Soluble solids (%) | Fruit hardness (kg/cm²) | Fruit brittleness (kg/sec) |
|----------------------|-------------------------|--------------------|-------------------------|---------------------------|
| Control              | 186.31±5.36             | 15.86±0.82         | 0.403±0.026             | 0.95±0.10                 |
| Treatment            | 200.36±6.25*            | 17.03±0.91*        | 0.396±0.034*            | 1.20±0.09*                |

Each treatment contained three replicates. * indicates significant differences between control and microbial agent treated fruits (P < 0.05).

3.1.2 Volatile Substances and Related Genes

35 volatile substances were detected in the treatment group and the control group, including esters, alcohols, aldehydes, and farnesene (Table 3, Figure 1A). The total volatile substances were mainly esters, accounting for 81.06% and 51.13% of the total volatile substances, respectively. 2-methylbutyric acid hexyl ester is the main ester substance, accounting for 19.17% and 26.32% of the ester content, respectively. The total volatile substances, esters, alcohols, aldehydes and farnesene in the treatment group were 31.13%, 7.8%, 6.73% and 34.06% higher than the control group, respectively.

The expression levels of five genes (AAT1, AAT2, LOX, HPL, ADH) related to aroma metabolism in the treatment group were higher than those in the control group (Figure 1B), which were 2.06, 2.3, 1.59, 1.22, 1.86 times of the control group, respectively.

It is indicated that microbial fertilizer has a significant effect on the improvement of fruit aroma substances, which is due to the regulation of the expression of related genes (AAT1, AAT2, LOX, HPL, ADH).

Table 3. Differences in the levels of volatile matter

| Volatile ingredient name | Control       | Treatment     | Volatile ingredient name | Control       | Treatment     |
|-------------------------|---------------|---------------|--------------------------|---------------|---------------|
| Ester                   | 6466.44±31.54 | 8479.32±37.55*| Amyl 2-methylbutyrate    | 29.37±1.33    | 34.04±2.57    |
| Butyl acetate           | 254.4±27.33   | 257.08±23.41  | Hexyl 2-methylpropionate | 15.58±1.34    | 20.84±1.43    |
| Ethyl butyrate          | -             | -             | Butyr acid-3-hexenyl ester | 4.81±0.19    | -             |
| Butyl 2-methylacetate   | 838.22±22.14  | 789.47±27.42  | Hexyl butyrate           | 826.173±22.8 | -             |
| Propyl butyrate         | 28.42±2.11    | 40.10±3.28    | Hexyl caproate           | -             | 1083.10±32.37 |
| Propyl propionate       | -             | -             | Butyl-2-butenyl ester    | 44.10±2.31    | 61.72±2.12    |
| Butyl propionate        | 35.29±2.45    | 41.86±3.15    | 2-methylbutyrate         | 1242.95±13.32 | 1356.03±14.54 |
| Butyl isobutyrate       | -             | -             | Butyl 2-methylhexanoate  | 46.79±1.56    | 53.06±2.33    |
| Amyl acetate            | 52.11±2.77    | 60.16±4.55    | Amyl hexanoate           | 54.55±3.32    | 73.53±3.31    |
| Propyl 2-methylbutyrate | 10.55±1.98    | 13.26±1.29    | Propyl octanoate         | 5.42±0.54     | 3.6±0.21      |
| Butyl 2-methylpropionat | -             | -             | 2-methylheptylbutyrate   | 3.50±0.38     | 3.49±0.42     |
| Compound                                    | Control    | Treatment   | p-value   |
|--------------------------------------------|------------|-------------|-----------|
| Butyl 2-methylbutyrate                     | 4.14±0.43  | 4.77±0.33   |           |
| 2-methyl-1-butanol propionate              | 13.06±0.82 | 12.88±0.27  |           |
| Butyl butyrate                             | 269.13±12.27 | 353.80±22.14 |           |
| Ethyl hexanoate                            | 54.60±4.34 | 81.26±6.42  |           |
| 2-methylbutyric acid                       | 8.41±0.32  | 10.94±0.54  |           |
| 2-methylpropyl ester                       |            |             |           |
| 2-hexen-1-ol acetate                       | 372.10±13.54 | 318.27±16.76 |           |
| 3-hexen-1-ol acetate                       | 31.87±1.54 | -           |           |
| 4-hexenyl propionate                       | -          | 19.86±2.51  |           |
| Hexyl acetate                              | 995.97±32.13 | 948.56±30.98 |           |
| Butyl 2-methylbutyrate                     | 275.16±17.86 | 223.49±15.33 |           |
| Butyric acid-pentyl ester                  | 47.07±4.32 | 64.41±6.71  |           |
| 2-methylbutyric acid-2-methylbutyl ester   | 49.24±3.19 | 37.24±1.28  |           |
| Hexyl propionate                           | 105.64±7.75 | 101.90±8.78 |           |
| 2-hexen-1-ol propionate                    | 14.22±1.32 | 16.43±1.54  |           |
| N-butyl butyrate                           | -          | -           |           |
| Heptyl hexanoate                           | 3.24±0.24  | -           |           |
| **Hexyl caproate**                         | 688.00±28.13 | 830.82±32.21 |           |
| **Octyl octylbutyrate**                    | 42.34±2.31 | 69.16±3.27  |           |
| **Hexyl octanoate**                        | -          | -           |           |
| **Alcohol**                                | 341.85±19.43 | 368.10±13.32* |           |
| **N-butanol**                              | 23.61±0.98 | 22.58±0.78  |           |
| **2-methylbutanol**                        | 39.80±1.04 | 30.95±0.94  |           |
| **Cyclohexanol**                           | -          | 54.04±2.43  |           |
| **1-hexanol**                              | 278.44±3.54 | 260.53±2.37 |           |
| **Aldehyde**                               | 289.58±12.75 | 309.07±8.09* |           |
| **Hexanal**                                | 86.14±3.19 | 89.72±3.14  |           |
| **2-hexenal**                              | 203.45±12.32 | 219.35±10.43 |           |
| **Hydrocarbon**                            | 1407.64±34.13 | 2134.79±27.31 |           |
| **Ethylbenzene**                           | 0.23±0.07  | 0.82±0.09   |           |
| **Tetradecane**                            | 7.32±0.91  | 32.75±1.23  |           |
| **Hexadecane**                             | 12.30±1.31 | -           |           |
| **Farnesene**                              | 1387.79±15.21 | 2101.22±12.21 |           |

The data in the table is the measured value per 100g fresh weight, and “-” means not detected.

Figure 1. Differences in the levels of volatile substances and expression of related genes. A, B, C, D, E, F stand for ester, alcohol, aldehyde, hydrocarbon, farnesene, respectively (A).
4. Discussion
The experiment found that the single fruit weight, soluble solids, fruit hardness, fruit brittleness, and cell structure of the microbial fertilizer treatment group were superior to the control group. The mechanism of action is that the microbial fertilizer increases the number of soil microbes, and the metabolites produced by the microorganisms enrich the soil nutrients and improve the soil environment [6]. We also found that the microbial fertilizer significantly increased the volatile matter content, because the microbial fertilizer up-regulated the expression levels of fruit AAT1, AAT2, LOX, ADH and HPL genes. Liu et al. also showed a positive correlation between volatile matter content and AAT1, AAT2, LOX, ADH, and HPL genes [4]. These above findings are consistent with the research results reported by Ren Xiuzhi, in which it was shown that microbial fertilizers significantly improve fruit quality, the intrinsic quality mainly improves the soluble solid content, sugar-acid ratio and aroma quality of the fruit, and the external quality improves the fruit coloration, hardness and fruit shape index [7]. The experiment results reported by Shao and Tan also revealed that the effect of microbial fertilizer on fruit quality improvement was obvious [8]. More studies have revealed that microbial fertilizer treatment increases the yield of apples by 114%, when compared with the control, and that the soil organic matter increased by 22%, when compared with the control [9]. Our study evidences the promoting effect of Pseudomonas fluorescens and Bacillus amyloliquefaciens co-utilization in improving fruit quality.

5. Conclusions
We found that that microbial fertilizer is effective in increasing the content of aroma and soluble solids, single fruit weight and brittleness, reducing the organic acid content of apple fruit. Our results provide novel evidence for agricultural science.

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