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Effects of wood vinegar on the soil microbial characteristics

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ABSTRACT

This project compares the dynamics of microbial quantities in the soil to analyze the impacts of diluted wood vinegar on soil microbial characteristics. In particular, the project adopts spraying treatments, respectively injecting 300-fold diluted wood vinegar (P300), 500-fold diluted wood vinegar (P500) and the same amount of water (CK) into three different plots. Then we adopt the phospholipid fatty acid (PLFA) method to measure the total microbial quantities, the total bacterial quantities, and the quantities of fungi and actinomycetes the soil, on the 1st, 3rd, 6th, 10th and the 15th day after the injection. The results showed that bacteria were the major microbial composition in the soil and that the dominant bacteria included bacillus spp. and Gram-positive bacteria. Treatments with wood vinegar at the two concentrations significantly increased the quantity of bacteria in soils and led to a significant increase in the total quantity of microbes. The P300 treatment changed the total number of bacteria mainly by changing the number of dominant bacteria, while the P500 treatment not only had a significant impact on the number of dominant bacteria but also showed strong effects on increasing the numbers of Gram-negative bacteria, anaerobic bacteria, aerobic bacteria and other non-dominant bacteria. There were smaller numbers of fungi and actinomycetes in the soils. While wood vinegar at the two concentrations exhibited certain inhibitory effects on soil fungi, the effects on actinomycetes need to be studied further.

Keyword: wood vinegar, phospholipid fatty acid, soil, soil bacteria, soil microbes

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INTRODUCTION

Wood vinegar is an organic liquid mixture produced through condensing the smoke produced during the carbonization or pyrolysis of wood and its residues from processing. The major composition of wood vinegar is acetic acid, and it also contains acids, alcohols, phenols, esters, carbonyl and furans and other organic ingredients. In the last century, a number of countries, such as Japan, have used wood vinegar in a wide range of applications, such as crop pest control, crop growth promotion, composting, deodorizing and feed additives[1]related research has also been conducted in these countries[2-11]. Yatagai M et al.[2]analyzed the composition of wood vinegar. Yoshimura H et a1.[3]studied the effects of wood vinegar mixtures on promoting fruit maturation. Ohta et al.[4]demonstrated that unrefined wood vinegar at certain concentrations was able to promote mycelium growth.

Soil microbes have important roles in plant growth; thus, it can be speculated that the demonstrated beneficial effects of wood vinegar on the growth, yield and quality of crops and compost fertilizer should be closely related to its effects on soil microbes. However, to date, few studies concerning this topic have been conducted. Nowadays the phospholipid fatty acids (PLFA) method is widely adopted in the study of soil microbial diversity. This method allows more comprehensive information about the soil microbial communities to be obtained [12-15]. Therefore, in the present study, the effects of applying wood vinegar at different concentrations on the soil microbes, bacteria, fungi and actinomycetes in the vegetable planting soil in the suburbs of Beijing were evaluated using the phospholipid

fatty acid (PLFA) method. Additionally, the characteristics of changes in bacillus spp., Gram gram-negative bacteria (G^-), Gram-positive bacteria (G^+), anaerobic bacteria (Ana), aerobic bacteria (Aer) and sulfur bacteria in the soil were further analyzed, providing a theoretical basis for future in-depth studies on the mechanisms of wood vinegar's effects on soils and plant growth.

EXPERIMENTAL SECTION

2.1 Study area

The study area is located in the Jinliuhuan vegetable planting area in the suburbs of Beijing. The topsoil texture is loam soil, the planting crop is tomato.

2.2 Experimental wood vinegar

The wood vinegar used for the experiment was purchased from Yixin Bioenergy Technology Development Co. Ltd., Shanxi. It is made from perennial oaks, and the main ingredients are shown in Tables 1 and 2.

Table 1 The main inorganic components in wood vinegar (mg.kg^{-1})

K	Na	Ca	Mg	Fe	Zn	Mn	Ni	Co	Al
13.23	146.15	375.88	133.43	578.62	16.71	7.46	0.05	0.003	2.78

Table 2 The main organic components in wood vinegar(%)

Acetic acid	Propionic acid	1-hydroxy-2-butanone	2-ethoxy-propane	Furfural	Butyrolactone	2-cyclopenten-1-ketone
2.87	0.41	0.84	0.08	0.69	0.77	0.09

2.3 Experimental design

In 2009-2010, the effects of six types of spraying treatments - CK, P200, P300, P400, P500 and P600 - on the root growth, yield and quality of tomatoes were compared (CK is the control treatment in which no wood vinegar was applied; P200 is to spray with 200-fold diluted wood vinegar that we purchased, and the same formula applies to other treatments), and it was found that P300 and P500 had the most significant effects on tomato growth. On this basis, the three treatments of CK, P300 and P500 were chosen for the present study, and there were three replicated plots (25m^2/plot) for each treatment. Four days after planting the tomatoes, each plot was sprayed with 1200 ml of wood vinegar at different concentrations. The CK cells were sprayed with the same quantity of water.

Fresh 0-20cm soil samples were collected from each plot 1, 3, 6, 10 and 15 days after spraying and were then mixed. The samples were stored at -20℃ and were later used to determine the quantity of soil microbes[16].

2.4 Measuring method and data analysis

2.4.1 Phospholipid fatty acid (PLFA) determination

After the sample preparation, An Agilent 6890 gas chromatograph (GC) and a mass spectrometer (MSD) Agilent 5973 were used in combination for the determinations[17-18]. Esterified 19:0 fatty acid with a calibrated concentration of 3.3 μg/mL was used as the internal standard. An HP-5 column (0.25 mm × 30 mm × 0.25 μm) was used. The column has an injection volume of 1.0 μL and a split ratio of 10:1. The carrier gas (N_2) flow rate is 0.25 mL/min, and the standby temperature is 50.0℃. The four-stage program of heating was 50℃ - 180℃ for 2 min, 12℃/min; 180℃ - 220℃ for 2 min, 6℃/min; 220℃ - 240℃ for 1 min, 15℃/min; and 240℃ - 260℃ for 15 min, 15℃/min. For flame ionization detector (FID) detection, the peak area was calculated by automatically integrating on the computer with manual correction/adjustment. The qualitative and quantitative analyses of fatty acids were performed in reference to the BAME (Bacterial Acid Methyl Esters) Mix and the Supelcoe 37 component FAME Mix, respectively[19-20].

2.4.2 Data processing

Variance analysis was performed on the indicator data using Excel 2010 and SPSS18.0 software. The phospholipid fatty acids (PLFAs) with contents less than 0.1% were excluded, and the results of soil microbial PLFA determination were then analyzed using Agilent MSDChem software and the NIST2005 sample structure database[21]. PLFA signatures serve to represent the specific soil microbial species (Table3), thus by summing up the amounts of PLFA signatures under the same representation we can obtain the microbial quantities in soil[22-28].

Table 3 Correspondence between PLFA markers and specific microbial species[29-36]

Type of microorganism indicated	PLFA marker
Total microbial quantity	The sum of all available PLFAs
Bacteria	14:0, 15:0, 16:0, 17:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0, 16:1ω7t, 18:1ω5, etc.
Bacillus spp.	Branched-chain fatty acids, etc.
G^-	Single-ene fatty acids, cyclopropyl fatty acids, etc.
G^+	Most are iso- and anteiso- branched-chain fatty acids
Ana	cy17:0, cy19:0, 18:1ω7c, 11:1ω3, etc.
Aer	16:1ω7, 16:1ω7t, 16:1ω9, 18:1ω7t, etc.
Sulfur bacteria	i17: 1ω5, 10Me18:1ω6, 11Me18:1ω6
Fungi	18:1ω9, 18:2ω6, 18:3ω6, 18:3ω3, etc.
Actinomycetes	10Me16:0, 10Me17:0, 10Me18:0, etc.

RESULTS

3.1 Effects of different treatments on total microbial quantity

There were no significant changes in the total microbial count of CK-treated soils, which fluctuated within the range of 5500-8000ng.kg^{-1}. However, P300 and P500-treated soils exhibited significant changes in microbial counts (Table 4). Three days after application, the total microbial count significantly increased in both types of soils, reaching their maxima at 11005.00ng.kg^{-1} and 18803.45ng.kg^{-1}, respectively. Although the total microbial counts of the P300 and P500-treated soils gradually decreased from that point, the total microbial counts remained at relatively high levels and significantly different from the microbial count of CK-treated soils up to 10 days after application; the total microbial counts of P300 and P500-treated soils decreased to the same level as the CK-treated soil 15 days after application.

After P300 and P500 treatment, the mean total microbial counts were 9224.38 ng.kg^{-1}and13105.71ng.kg^{-1}, respectively. These counts were greater than the CK treatment by 19.37% and 69.59%, respectively; 3 days after application, the differences reached 49.21% and 154.95%, respectively. Compared to P300 treatment, P500 treatment exhibited a stronger effect on the total soil microbial count.

Table 4 Total quantities of soil microbes at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	8033.98（16.58）a	11005.00（13.49）b	10131.74（19.54）b	8924.46（25.17）b	8026.76（14.34）a
P500	9527.13（22.49）a	18803.45（46.52）a	14828.22（58.17）a	14114.42（23.49）a	8255.35（29.36）a
CK	7723.74（64.34）a	7375.33（36.24）c	8471.51（44.69）c	7048.18（78.69）c	8020.13（33.95）a

Note: a, b and c are the levels of count difference between the three treatments (P <0.01) and are the same hereafter.

3.2 Effects of different treatments on bacterial quantities

3.2.1 Effects of different treatments on the total bacterial quantity of soils

On average, bacterial quantities in soils treated with CK, P300 and P500 were 7079.11 ng.kg^{-1},8592.20 ng.kg^{-1}and12174.66 ng.kg^{-1}, accounting for 91.61%, 93.15% and 93.66% of total microbes, respectively, suggesting that bacteria were the most important microbial soil components (Table 5).

The characteristics of the changes in bacterial quantities were fully consistent with the changes in total microbial quantities in both P300- and P500-treated soils: compared with CK-treated soils, the soil bacteria quantities increased significantly by 21.37% and 73.39%, respectively. Wood vinegar-treated soils exhibited similar phenomena: they did not significantly differ from CK-treated soils in terms of bacterial quantities on day 1, and quantities quickly reached the maximum on day 3. Then, despite gradually decreasing, bacterial quantities returned to the level of CK-treated soils until day 15. Compared to the P300 treatment, the effect of P500 treatment on increasing soil bacteria quantity was more significant: P500-treated soils had significantly higher quantities of bacteria than did P300-treated soils on days 3, 6 and 10 (Table 5).

Table 5 Soil bacteria quantities in soils at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	7233.13（22.42）a	9716.86（68.15）b	10049.05（22.45）b	8235.41（34.51）b	7726.55（41.23）a
P500	9017.74（41.13）a	16906.74（55.42）a	13779.13（43.36）a	13414.26（62.16）a	8255.41（34.24）a
CK	7458.62（11.18）a	6575.41（24.22）c	7258.42（19.46）c	6648.87（34.48）c	7454.23（9.14）a

3.2.2 Effects of different treatments on major soil bacteria

1）Characteristics of changes in bacillus spp.

P300 and P500 treatment had very strong effects on the quantities of soil bacillus spp.: the differences on days 3, 6 and 10 were significantly different compared to CK treatment, and the quantities increased significantly. In particular, the effects of P500 treatment were more significant: the quantity of soil bacteria significantly increased, reaching 3509.59ng.kg^{-1}; in the three measurements afterwards, bacterial quantities were still significantly higher compared to the CK treatment by 3441.01ng.kg^{-1}, 3702.79 ng.kg^{-1} and 3822.17 ng.kg^{-}, respectively (Table 6).

Under the CK, P300 and P500 treatments, the average quantities of soil bacillus spp. were 2217.85 ng.kg^{-1}, 3063.99 ng.kg^{-1} and 4596.25 ng.kg^{-1}, accounting for 31.33%, 35.66% and 37.45% of the total bacteria quantities, respectively.

Table 6 Quantities of soil bacillus spp. at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	2552.41（10.36）b	3310.78（21.14）b	4041.34（26.36）b	3225.17（17.08）b	2260.26（4.38）a
P500	3509.59（22.82）a	5756.27（42.64）a	5851.13（37.38）a	5523.25（21.21）a	2341.00（10.00）a
CK	2556.44（33.47）b	2315.26（21.21）c	2148.34（18.34）c	1701.08（10.72）c	2368.13（13.18）a

2）Effects on soil Gram-positive bacteria

The P300 and P500 treatments also showed strong effects on the quantity of G^+ in soils, with trends in quantity changes very similar to bacillus spp. during the 15-day experimental period. The quantity of G^+ in the P500-treated soils significantly increased the first day after application and significantly differed from the quantity in CK-treated soils. The quantity of G^+ in P300-treated soils began to increase rapidly on days 3, 6 and 10. Although the increase was lower than that in the P500-treated soils, the differences when comparing P200- to CK-treated soils were highly significant. On day 15, the effects on G^+ by P300 and P500 treatments disappeared, and the quantities returned to the level as CK treatment (Table 7).

Similarly to bacillus spp., the quantities of G^+ in soils accounted for large portions of bacteria: the average quantity of G^+ (over 5 measurements) in the CK-, P300- and P500-treated soils accounted for 31.47%, 36.18% and 37.91% of the average total bacterial quantities, respectively.

Table 7 Quantities of soil Gram-positive bacteria at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	1655.36（35.45）b	3310.57（22.20）b	4041.31（10.46）b	3625.19（20.04）a	2910.07（5.93）a
P500	3509.58（22.74）a	5756.61（36.23）a	4551.16（14.26）a	6104.96（17.34）a	3341.24（10.05）a
CK	2556.69（31.18）b	2315.85（27.73）c	2148.14（15.74）c	1601.58（10.94）b	2515.74（21.67）a

3）Effects on soil Gram-negative bacteria

P300 treatment did not show large effects on the G^- in soils, which were characterized by changes in bacterial quantity similar to CK treatment, with no significant difference in the quantities. However, P500 treatment exhibited strong effects: the bacterial quantity significantly increased on day 3, reaching 2461.27ng.kg^{-1}. Although the quantity decreased to 1640.28ng.kg^{-1} at day 6, the difference compared to CK treatment was still significant. The quantity returned CK treatment levels on day 10 (Table 8).

There was a small number of G^- in soils, accounting for a small percentage of the total quantity of bacteria: 9.27% (CK), 8.07% (P300) and 9.32% (P500).

Table 8 Quantities of soil Gram-negative bacteria at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	992.46（14.45）a	1037.39（9.13）b	767.44（16.62）b	540.22（8.48）a	131.37（7.14）a
P500	898.16（16.12）a	2461.27（11.54）a	1640.28（8.42）a	650.47（11.69）a	72.00（5.69）a
CK	992.21（1.18）a	897.22（6.64）b	750.51（12.84）b	590.97（2.47）a	50.21（9.23）a

4）Effects on soil anaerobic bacteria

P300 treatment showed no significant effects on Ana quantity in soils. However, P500 treatment showed strong effects: the bacterial quantity reached a maximum of 1493.87 ng.kg^{-1} on day 3; although the quantity subsequently rapidly decreased to 710.78 ng.kg^{-1} and 475.78 ng.kg^{-1}, it was still significantly different compared to CK treatment; on day 15, the effect disappeared, and the bacterial quantity returned to the level as in the CK-treated soils. (Table 9)

The small quantity of Ana in the soils only accounted for 3.62% (CK), 3.26% (P300) and 5.25% (P500) of the total bacterial quantity.

Table 9 Quantities of soil anaerobic bacteria at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	397.13（4.42）a	227.22（6.21）b	333.71（7.55）b	164.58（8.06）b	278.12（5.34）a
P500	390.36（5.52）a	1493.87（15.36）a	710.78（17.74）a	475.78（10.47）a	153.20（6.50）a
CK	201.41（2.30）a	186.75（5.25）b	413.39（8.48）b	268.14（6.15）b	213.13（4.31）a

5）Effects on soil aerobic bacteria

P300 treatment exhibited no significant effects on the quantity of Aer in soils. However, with P500 treatment, the bacterial quantities increased significantly on days 1, 3 and 6, indicating strong effects (Table 10).

The quantity of Aer-3% for all the three types of treatments - only accounted for small percentages of the bacterial populations in soils.

Table 10 Quantities of soil aerobic bacteria at different times (ng.kg)

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	280.25（8.68）b	250.57（0.44）b	434.26（6.71）b	186.27（3.13）a	210.41（9.65）a
P500	407.47（3.65）a	406.51（4.15）a	930.39（8.24）a	175.47（3.55）a	178.05（3.54）a
CK	166.29（10.36）b	120.34（3.25）b	338.41（14.04）b	231.36（4.62）a	180.48（8.81）a

6）Effects on soil sulfur bacteria

The quantities of sulfur bacteria in soils were the lowest and could not be detected in nearly 50% of the samples. Therefore, it is difficult to accurately determine wood vinegar's effect on sulfur bacteria. However, according to the available data, it appears that wood vinegar treatment still had some stimulating effect on its quantity. Particularly on day 3, the quantities of sulfur bacteria in soils treated with wood vinegar at the two concentrations were significantly higher compared to CK-treated soils (Table 11).

If not detected, the quantity of sulfur bacteria was treated as 0 in the calculations. This type of bacteria accounted for only 1-2% of the total bacteria populations in soils.

Table 11 Quantities of soil sulfur bacteria at different times (ng.kg^{-1})

Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
P300	225.69（1.31）a	502.11（0.94）a	156.25（5.25）b	143.58（4.51）	-
P500	182.12（7.08）a	434.36（1.56）a	-	-	-
CK	-	115.27（5.03）b	384.12（7.24）a	-	-

Note: "-" means the quantity of sulfur bacteria was too low to be detected.

3.3 Effects of different treatments on the quantities of fungi and actinomycetes in soils

The numbers of fungi in the soils were low and were not detected in some of the samples. If not detected, the quantity of fungi in soils was treated as 0 in the calculations. Under the CK, P300 and P500 treatments, the average quantities of fungi in soils were only 300.59ng.kg^{-1}, 185.84ng.kg^{-1} and 184.26ng.kg^{-1}, accounting for 3.89%, 2.01% and 1.41% of the total microbial quantities, respectively. The average quantities of fungi were almost the same in the P300- and P500-treated soils, both being approximately 38% less than the quantity in CK-treated soils (Table 12).

Table 12 Quantities of soil fungi and actinomycetes at different times (ng.kg^{-1})

Type	Treatment	Day 1	Day 3	Day 6	Day 10	Day 15
Fungi	P300	-	432.11（5.48）a	164.45（7.71）a	332.63（6.37）	-
	P500	209.17（8.82）a	362.58（10.64）a	349.68（12.41）a	-	-
	CK	624.32（12.85）a	-	412.28（7.94）a	-	466.33（11.06）
Actinomycetes	P300	-	354.06（6.33）a	217.25（4.15）	156.39（8.56）	-
	P500	-	433.11（11.94）a	-	-	-
	CK	-	-	-	-	-

The quantity of actinomycetes was the lowest in soils; actinomycetes were detected in only 27% of the samples. Actinomycetes were not detected in all CK-treated soils during the entire experiment period. Although actinomycetes were detected in some of the P300- and P500-treated samples and it appears that the treatments had

certain stimulating effects on actinomycetes, the detection rate was too low to draw definite conclusions (Table 12).

DISCUSSION AND CONCLUSION

4.1 Bacteria are the dominant component of soil microbes; their quantities account for 90% of the total numbers of microbes. The two types of wood vinegar treatments, P300 and P500, exhibited strong promoting effects on the total microbial quantities in soils. These effects were achieved mainly by changing the numbers of bacteria.

4.2 Wood vinegar can cause significant increases in the quantities of bacteria in soils. Under P300 and P500 treatments, the quantities of soil bacteria were 21.37% and 73.39% higher than the quantities under CK treatment, respectively, while they showed a certain degree of inhibition on soil fungi, reducing the quantities by approximately 38%. Studies have shown that the bacteria-to-fungi ratio can, to a certain level, reflect the sustainability and stability of the soil ecosystem: a lower ratio indicates higher sustainability and stability of the soil system [37]. In the CK-, P300- and P500-treated soils, the bacteria-to-fungi ratios were 23.55, 46.23 and 66.07, respectively; Relative to CK, the treatments of P300 and P500 increases the bacteria-to-fungi ratios by 96.31% and 180.55%, respectively. As a result, by increasing bacterial quantities and inhibiting actinomycetes, wood vinegar dramatically changes the sustainability and stability of the soil ecosystem Evidence of applications shows that wood vinegar has good control effects against Rhizoctonia solani, Botrytis cinerea, powdery mildew and rot, and this should be closely related with its the effects on soil ecosystem especially through the inhibition of the actinomycetes.

4.3 Wood vinegar did not change the characteristics of the soil bacterial composition. Under all three types of treatment – CK, P300 and P500 – bacillus spp. and G^+ were still the dominant bacteria, accounting for 62.80% 71.84% and 75.36% of the total quantity of bacteria, But we should also note that the two treatments increase the proportion of the dominant bacteria in soil to some extent as well. respectively, while the G^-, Ana, Aer and sulfur bacteria accounted for small proportions of the total quantity, without the quantitative characteristics of the dominant bacteria.

4.4 The populations of the dominant soil bacteria species were strongly affected by wood vinegar application. Wood vinegar at both concentrations could significantly increase the quantities of bacillus spp. and G^+ in soils, but their effects on the quantities of non-dominant species were related to the concentration of applied wood vinegar. With P500 treatment, the quantities of G-, Ana and Aer in soils significantly increased, while under P300 treatment, the quantities of non-dominant bacteria did not significantly differ compared to quantities with CK treatment. Due to the small quantity of sulfur bacteria in soils, the effect of wood vinegar on their population could not be determined.

G^+/G^- can reflect the sensitivity of G^+ and G^- to environmental stimuli; thus, a dramatic change in the ratio could mean changes in the microbial community structure in the soil ecosystem. Under the CK, P300 and P500 treatments, the values of G^+/G^- were 3.09, 4.48 and 4.06, respectively. The difference between the three treatments did not reach the significant level. Therefore, although the application of wood vinegar could significantly increase the numbers of bacteria in soils, it had little effect on the community structure.

Aer/Ana reflects the relative compositions and environmental sensitivities of Aer and Ana in soils. Under the CK, P300 and P500 treatments, the Aer/Ana values were 0.83, 0.97 and 0.98, respectively, and were not significantly different from one another.

4.5 The two types of wood vinegar treatment could cause the quantities of microbes in soils to increase rapidly on days 1 or 3 after application. However, the increases were only sustained for short periods of time, usually 7-10 days, and the increased quantities were related to the applied wood vinegar concentrations and the types of microbes.

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