Influence of Fertilizer pH and Presence of FOC on Soil Microbial Functional Diversity

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Abstract: Background and objective: Fusarium wilt (Panama disease) of banana is caused by the soil-borne fungus Fusarium oxysporum f. sp. cubense (FOC), whose proliferation is favored under acidic soil conditions. This study investigated whether soil microbial functional diversity is associated with interactions between fertilizer pH and FOC spore inoculate. Method: Pseudostems (n = 180) were grown in pots in mixed soil (pH 6. 09). In a 3×5 design, each pot was inoculated with 0, 103, or 106 cfu FOC/g of soil, and irrigated each week with pH 5, 6, 7, 8, or 9 fertilizer; N, P2O5, and K2O were identical. At 65 days, spore suspensions were prepared from soil samples. Community level physiological profiling (CLPP) was conducted, using the Biolog EcoPlate method, through indices of carbon source utilization that reflect the metabolic functional diversity of soil microbes: average well color development (AWCD), richness (R), Shannon-Weaver diversity index (H'), and the Simpson dominance index (D). Principal component analysis (PCA) of the functional diversity of microbes was applied. Results: AWCD was significantly higher in treatments consisting of no FOC inoculate and alkaline fertilizer compared with other treatments, and R, H', and D were also notably higher (72 h). Results of PCA revealed that the diversity of soil microbes was higher under alkaline fertilizer treatment, but lower under conditions of FOC inoculation. Conclusions: The application of alkaline fertilizer increases the functional diversity of soil microbial populations.

1. Introduction

Banana production is greatly threatened worldwide by Fusarium wilt (Panama disease) of banana, a disease of the vascular tissue. The causative agent of Fusarium wilt of banana is the soil-borne fungus Fusarium oxysporum f. sp. cubense (FOC) [1]. The fungus can survive for a long time even in severe environments. Fusarium wilt is especially problematic in areas where soils are poor in nutrients, organic matter, texture, and with low pH (<6. 0) [2]. To avoid infection, bananas have been planted in regions where they have rarely grown in history.

Fusarium wilt disease is currently the most important threat to China’s banana industry, a situation made worse by the lack of disease-resistant cultivars and the injudicious use of fertilizers that lead to soil acidification. In fact, the main cause of Fusarium wilt outbreaks is soil acidification caused by the long-term use of chemical fertilizers or their overuse, because low soil pH favors the proliferation of FOC over competing actinomycetes and bacteria [3-6]. Because a disturbance in the natural micropopulation of the soil is associated with disease [7], a potential strategy for disease control is to encourage and restore the soil’s microbial diversity [8]. Another method is to increase soil pH with alkaline fertilizer—such treatment during banana growing season has proved to lower remarkably the disease index and incidence of FOC, and increasing the harvest of banana fruit by as

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much as 20% [3]. However, there is no direct evidence that the application of alkaline fertilizer can affect the diversity of the soil microbial community.

This study investigated whether soil microbial functional diversity is associated with interactions between fertilizer pH and FOC spore inoculate.

2. Materials and Methods

2.1 Experimental Materials.

The soil used in these experiments was a 1:1 volume mix of sandy soil from a farm at South China Agriculture University combined with Gro-Med substrate (Vaighai Agro Products, Madurai, India). The pH of the mixed soil was 6.09. The mixed soil contained 17.09 g•kg-1 organic matter (determined by the potassium bichromate-dilution heat colorimetric method), 56.59 mg•kg-1 available nitrogen (via Kjeldahl digestion), 59.57 mg•kg-1 available phosphorus (extracting solution of 0.03 N NH4F in 0.025 N HCl), and 315.4 mg•kg-1 available potassium (extracting solution of 1 N ammonium acetate/acetic acid).

Sterile tissue-cultured Musa AAA Giant Cavendish cv. Baxi plants with 6 leaves were used for the experiments.

Spores of Fusarium oxysporum f. sp. cubense (E. F. Smith) Snyder and Hansen (FOC) was supplied by the Plant Pathology Lab of the College of Natural Resource and Environment of South China Agriculture University. The concentration of the FOC spore suspension was 1 × 107 cfu•mL-1.

Conventional fertilizers are pH 5 to 7, and alkaline fertilizers are pH 8 and 9. The experimental treatments used in the present study consisted of 5 compound fertilizers, 22N:8P:15K, with pH values 5, 6, 7, 8, and 9 (Guangdong Provincial Research Center of Environment Friendly Fertilizer Engineering & Technology).

The experiment was performed in a greenhouse at South China Agricultural University (latitude 23°09′40″ N, longitude 113°21′28″ E) on 4 October 2013.

2.2 Study Design.

This experiment was a 3×5 completely randomized design, that is, FOC concentration × fertilizer pH value. Thus, there were 15 treatments. Specifically, the tested FOC concentrations were 0, 103, and 106 cfu per gram of soil, termed C1, C2, and C3 respectively. The pH values of the compound fertilizers applied to the experimental soils were pH 5-pH 9, termed F1-F5 respectively. In each treatment replication, the doses of N, P2O5, and K2O were identical.

Each pot was 15 cm × 13 cm × 12 cm and contained 500 mg of the mixed soil with one banana pseudostem; each pseudostem had at least 6 leaves at the start of the experiment. The C1, C2, and C3 FOC spore suspension treatments were added to the 500 mg of mixed soil around each banana pseudostem from a syringe containing 50 mL of 0, 1 × 104, or 1 × 107 cfu•mL-1, respectively.

Fifty milliliters of 0.6% fertilizer solution were irrigated into each replication every week. One individual plant was considered a single replication in each treatment, and each treatment was replicated 12 times. Thus, there were 15 treatments and 180 plants in this study.

2.3 Sample Collection.

Sixty-five days after the inoculation of the FOC spore suspension, soil samples were collected from each of the 12 replicates in each of the 15 treatments. For each treatment, 3 sets of soil samples were created by randomly combining 4 replicates. The soil samples were stored at 4 °C.

2.4 Biolog EcoPlate Method.

The Biolog technique can be used to investigate the metabolic functional diversity of soil microbes as reflected by the utilization ratio of carbon sources [9, 10]. The protocols for creating serially diluted spore suspensions of the soil samples and the Biolog EcoPlate method were performed in accordance with Classen et al.’s methods [11]. Briefly, the soil samples are placed into an incubator at 25 °C for 24 hours. The water content of each fresh soil sample was determined, and

the fresh soil equivalent of 10 g of dry soil was put into a 250-mL triangular flask with 100 mL of sterilized saline (0.85%). After shaking for 30 min, 3 mL of supernatant was collected and diluted with 27 mL of sterilized saline in serial dilutions of 10× and 100×. The 150 μL of 100×-diluted solution was added into each hole of a Biolog EcoPlate, which then was kept at 25 °C.

Each Biolog EcoPlate consisted of 3 identical sections (replicates), each section containing 31 micro wells with distinct dissolved organic carbon substrates, and one well with no substrate to serve as negative control (blank; Table 1). For analysis, each plate was treated as 3 individual soil samples for the same treatment was used to fill all wells of the plate. Three replicates in one EcoPlate for each treatment were detected. The optical densities and average well color development (AWCD) in the inoculated Biolog EcoPlate was measured at culture times 3, 24, 36, 48, 72, 96, 120, 144, and 168 h, using a ELx808-Biolog absorbance reader (BioTek Instruments, USA) under a light wavelength of 590 nm.

Table 1 Carbon substrates of the Biolog EcoPlate wells. Each Biolog EcoPlate contains 96 wells consisting of three replicates (first 3 columns) of 31 substrates and 1 water blank.

96 EcoPlate cell			Substrate	Substrate ID
A1	A5	A9	None(water blank)	None
A2	A6	A10	β-Methyl-D-glucoside	1
A3	A7	A11	D-Galactonic acid γ-Lactone	2
A4	A8	A12	L-Arginine	3
B1	B5	B9	Pyruvic acid methyl ester	4
B2	B6	B10	D-Xylose	5
B3	B7	B11	D-Galacturonic acid	6
B4	B8	B12	L-Asparagine	7
C1	C5	C9	Tween 40	8
C2	C6	C10	I-Erythritol	9
C3	C7	C11	2-Hydroxy benzoic acid	10
C4	C8	C12	L-Phenylalanine	11
D1	D5	D9	Tween 80	12
D2	D6	D10	D-Mannitol	13
D3	D7	D11	4-Hydroxy benzoic acid	14
D4	D8	D12	L-Serine	15
E1	E5	E9	α-Cyclodextrin	16
E2	E6	E10	N-Acetyl-D-glucosamine	17
E3	E7	E11	γ-Hydroxybutyric acid	18
E4	E8	E12	L-Threonine	19
F1	F5	F9	Glycogen	20
F2	F6	F10	D-Glucosaminic acid	21
F3	F7	F11	Itaconic acid	22
F4	F8	F12	Glycyl-L-glutamic acid	23
G1	G5	G9	D-Cellobiose	24
G2	G6	G10	Glucose-1-phosphate	25
G3	G7	G11	α-Ketobutyric acid	26
G4	G8	G12	Phenylethylamine	27
H1	H5	H9	α-D-Lactose	28
H2	H6	H10	D, L-α-Glycerol phosphate	29
H3	H7	H11	D-Malic acid	30
H4	H8	H12	Putrescine	31

2.5 Statistical Analyses.

Individual plate readings were exported into Microsoft Excel for compilation and manipulation. Data from each plate were parsed into 3 data lines for the 3 replicates. For each plate, the optical

density (OD) of the blank well was subtracted from the OD of each of the 31 wells of its replicate to calculate a net substrate (normalized) OD value [12]. When blanking resulted in negative values, these values were set to zeros for data analysis [13].

The optical densities and AWCD of the wells in the Biolog EcoPlate are considered positively associated with the utilization of carbon sources, and therefore reflect the activity of soil microbes. The AWCD conforms to a time-dependent asymptotic sigmoidal curve [14]. For each replicate reading, the AWCD was calculated as the sum of all blanked substrate OD values, divided by 31 [15], as follows:

AWCD= ∑(ODi-R)/n, where ODi represents the OD value of each well of the substrate, R represents the optical density of the control (blank), ODi-R is the corrected absorbance value, and n is the 31 types of carbon substrates [12, 13].

The richness (S) measurement is the number of carbon substrates metabolized by the microbial community, that is, the corrected absorbance value (ODi - R) > 0.25 [16-18].

The Shannon-Wiener diversity index (H') was calculated as:

H' = -∑(Pi•logPi), where Pi = (ODi - R)/∑(ODi - R), and Pi is the proportional optical density value of each well [19, 17].

A large H' value indicates higher functional diversity [20].

The Simpson dominance index (D) was calculated as:

D = 1-∑Pi2. The Simpson's diversity index represents the probability that two individuals randomly selected from a sample will belong to different species, hence the greater the D-value (in which the maximum value is one), the greater the sample diversity [16].

The principal components analysis (PCA) of the OD values at 72 hours of cultivation time was performed with ADE-4 Software [21] to compare the carbon substrate utilization profiles by the FOC inoculation and fertilizer pH treatments. A permutation test was used to test the significance of the groupings suggested by the PCA. Values were considered as different when the probability to reject the nil hypothesis was lower than 0.05 [22]. Duncan's multiple range tests were performed using SAS statistical software.

3. Results

Utilization of Carbon Sources by Soil Microbes. In general, carbon utilization by the microbial population in this study increased during incubation (Fig. 1). The AWCD curves showed that, for all experimental treatments, carbon utilization increased slowly in the first and last 24 h, rapidly from 24 to 96 h, and was fairly stable from 96 to 144 h. An analysis according to FOC inoculate concentration showed that, during the interval of greatest change, carbon utilization in soils inoculated with either 103 cfu•g-1 (C2) or 106 cfu•g-1 (C3) was significantly lower than that of soils that received no FOC spores (0 cfu•g-1; C1; Fig. 1a). Similarly, carbon utilization was significantly and positively associated with fertilizer pH, from pH 5 (F1) to pH 9 (F5; Fig. 1b).

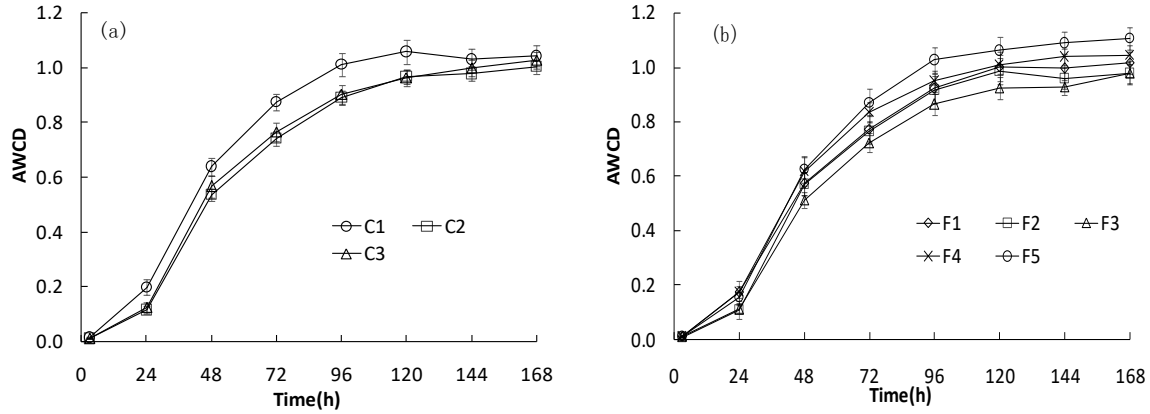

Figure. 1 AWCD of all carbon sources by cultivation time.

The 31 types of carbon sources tested in the Biolog EcoPlate consist of carboxylic acids, amino

acids, carbohydrates, polymers, phenols, and amine groups. In the C2 and C3 groups, the utilization of carboxylic acids, amino acids, carbohydrate, polymers, and amines was lower than in the C1 group (Fig. 2a). In contrast, in the C3 group, the utilization of phenols was higher than in the C1 and C2 groups.

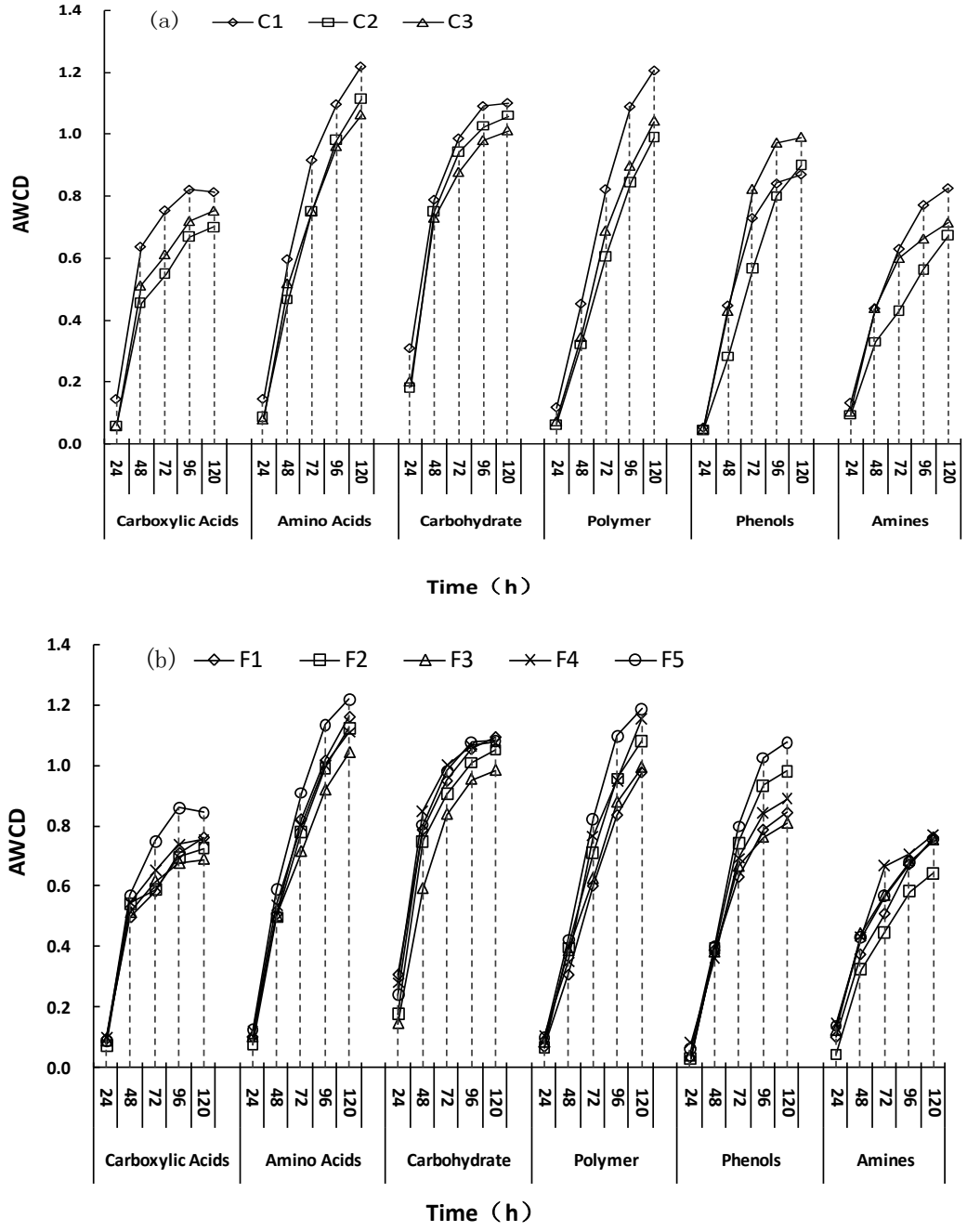

Figure. 2 AWCD by carbon source. (a) C1, C2, and C3 treatments; (b) F1, F2, F3, F4, and F5 treatments

The utilization of each carbon type was significantly greater in the F5 group than in the F, F2, F3, or F4 groups (Fig. 2b).

3.1 Functional Diversity of Soil Microbial Communities

The greatest difference in AWCD was recorded at the 72-h timepoint of the incubation period (Fig. 2). Therefore, metabolic functional diversity was analyzed according to the AWCD, richness (S), Simpson dominance index (D) and Shannon-Wiener diversity index (H') at the 72-h timepoint for each treatment (Table 2). The results showed that the AWCD, S, D, and H' of the C1 group were significantly greater than these indicators in the C2 and C3 groups. This suggests that soil microbial

diversity and metabolic activity was much higher in treatments that did not contain FOC inoculate compared with those that did.

Considering the fertilizer treatments, AWCD, D, and H' were significantly higher in the F5 (pH 9) groups than in the F1, F2, or F3 (pH 5-7; Table 2). Moreover, the richness in the F5 group was significantly greater than that in the F3 and F1 groups. AWCD and S in the F4 group (pH 8) were significantly higher compared with the F3 (pH 7) group. Thus, the soil microbial diversity and metabolic activity were highest in treatments receiving the alkaline fertilizer solutions.

3.2 PCA of the Functional Diversity of the Soil Microbial Community

PCA was conducted to understand better the functional diversity of microbes in the soil microenvironment [23] in response to the different treatments, and with the utilizing profiles of 31 types of carbon substrates for the different treatments to analyze associations among these carbon sources (Fig. 3 and 4).

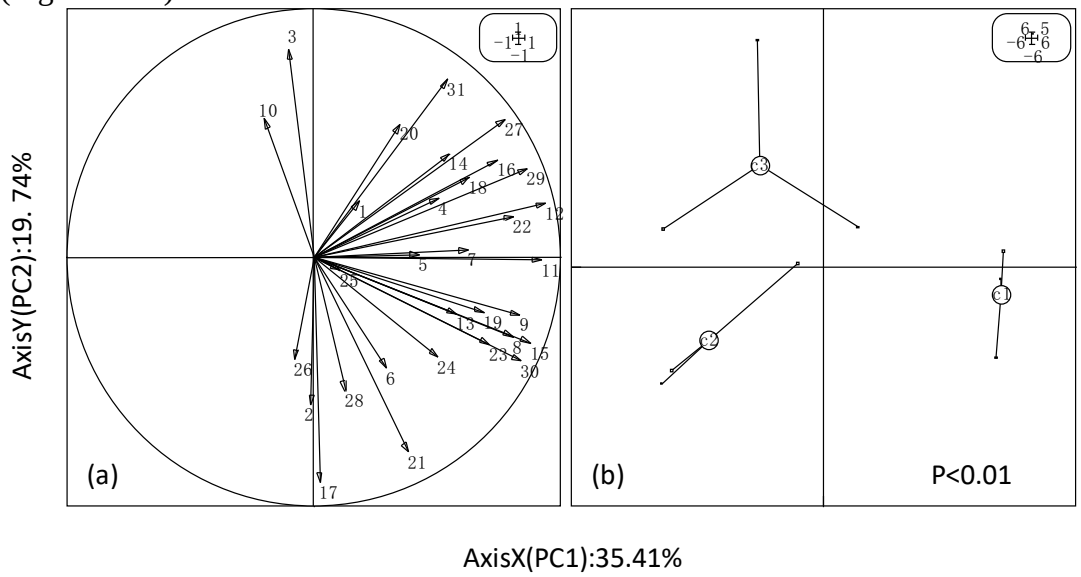

Figure. 3 PCA of the functional diversity of soil microbes of C1, C2, and C3 treatments.

(a)Loading plots of principal components 1and 2; (b) Distribution of scores of AWCD on PC1 and PC2. Note: The key for the identification numbers of the 31 substrates are listed in Table 1.

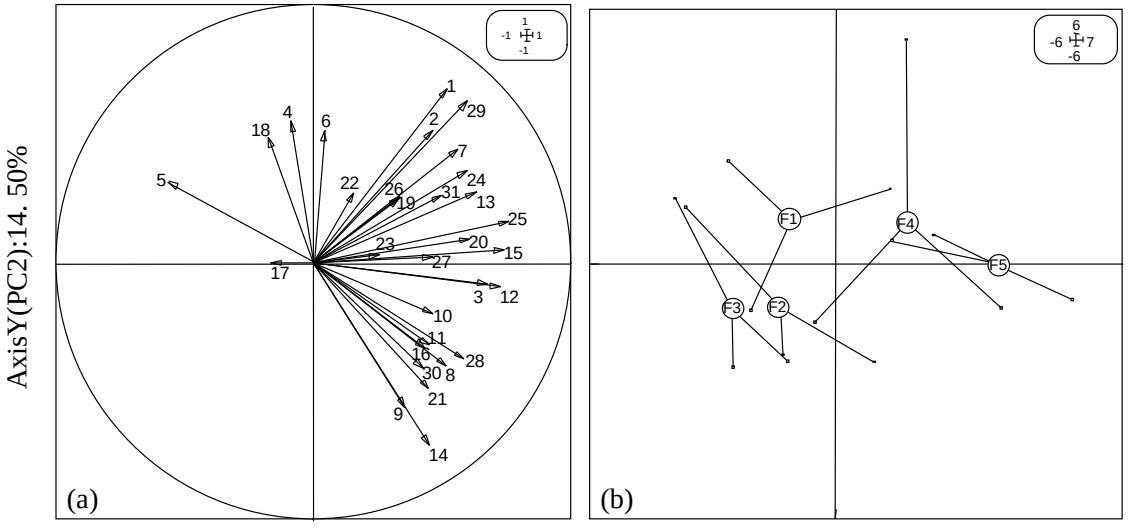

Figure 4. PCA of the functional diversity of soil microbes of F1, F2, F3, F4, and F5 treatments

(a) Loading plots of principal components 1 and 2; (b) Distribution of scores of AWCD on PC1 and PC2. Note: Note: The key for the identification numbers of the 31 substrates are listed in Table 1.

To distinguish the effects of the C1, C2, and C3 treatments on soil microbial catabolic diversity, a PCA was performed (Fig. 3a and b). The results showed that the first principal component (on the figure, axis X[PC1]) was mainly and positively determined by the utilization rates of Tween 80 (12), L-phenylalanine (11) and L-serine (15), and mainly negatively determined by the utilization rates of 2-hydroxybenzoic acid (10); its relative inertia was 35. 41%.

The C1 treatment was clearly distinguished from C2 and C3 by their respective carbon substrate utilization profiles, with different utilization rates (Fig. 3b): C1 had higher utilization rates for most carbon sources than did C2 and C3, especially for Tween 80, L-phenylalanine and L-serine. By contrast, its utilization rate for 2-hydroxybenzoic acid was considerably lower than those of C2 and C3.

The second principal component (Fig. 3; axis Y[PC2]) was mostly related to L-arginine (3), N-acetyl-D-glucosamine (17), D-glucosaminic acid (21); its relative inertia was 19.74%. C2 and C1 had higher utilization rates for N-acetyl-D-glucosamine (17) and D-glucosaminic acid (21) than did C3. However, their utilization rates for L-arginine (3) were considerably lower than that of C3. Therefore, C3 could be significantly differentiated from C2 and C1 in the second principal component. The permutation test of the PCA showed that the differences in the carbon substrate utilization profiles among C1, C2, and C3 were significant ($P < 0.00001$).

The carbon substrate utilizing profiles of the F1, F2, F3, F4, and F5 groups were also analyzed according to the PCA (Fig. 4a and b). The results showed that the first principal component was mainly and positively determined by the utilization rates of glucose 1-phosphate (25), L-serine (15), and Tween 80 (12), and mainly negatively determined by the utilization rates of D-xylose (5); its relative inertia was 29. 05%. The carbon substrate utilizing profiles of the conventional fertilizers (F1, F2, F3) were clearly different from that of the alkaline fertilizers (F4, F5; Fig. 4b): F4 and F5 had higher utilization rates for most carbon sources than did F1, F2, and F3, especially for glucose 1-phosphate, L-serine, and Tween 80. By contrast, their utilization rates for D-xylose were considerably lower than those of F1, F2, and F3.

4. Discussion

This study investigated whether soil microbial functional diversity may be augmented under specific fertilizer pH and FOC spore inoculate conditions. In a 3×5 completely randomized study design, banana pseudostems were individually grown in soils inoculated with 0, 103, or 106 cfu FOC/g of soil (FOC treatments C1, C2, and C3, respectively) and irrigated with fertilizer solutions of pH 5, 6, 7, 8, or 9 (pH treatments F1-F5). CLPP was conducted after 65 days using spore suspensions prepared from the soil and the Biolog EcoPlate method. The metabolic functional diversity of soil microbes was measured by the indices AWCD, S, H', and D, and then PCA was conducted. The results suggest that, overall, carbon utilization was lower when FOC inoculate was present, and soluble alkaline fertilizer (pH 8 or 9) was associated with greater soil microbial diversity and carbon utilization.

Some studies have shown that the overuse of inorganic fertilizer can damage the habitat and disrupt the function of soil microbial communities [24]; the injection of urea and ammonia in bands reduced total microbial activity [25]. My previous study [3] also showed that the over-application of conventional fertilizers (pH 7 or lower) led to soil acidification and an imbalance of microbes in the soil, because neutral or slightly alkaline soil conditions favored bacterial and actinomycete growth. Conversely, an acid pH favored fungal growth [26]. A possible reason is that FOC can adapt better than other microbes to acid soil and therefore can out-compete them. That is, FOC proliferation in soils made acid by conventional fertilizer may inhibit the carbon utilization of other soil microbes, thereby altering the soil microbial environment [27], while the application of alkaline fertilizers may enhance the microbial utilization rates of carbon sources. The present study is consistent with these previous studies.

However, the mechanism whereby alkaline fertilizer may benefit soil microbial diversity is unclear. It may be that the lower pH inhibits the activity of FOC, which benefits the utilization of carbon sources by other microbes. To control Fusarium wilt of banana, some researchers have tried

regulating the soil pH of degraded orchards [2] or found that the application of nitrate nitrogen fertilizer (physiologically alkaline) was better able to inhibit the growth of the pathogen than the use of ammonium nitrogen [28-33]. Another reason may be that actinomycetes and bacteria prefer neutral and slightly alkaline soils [26]. Our study indicated that the diversity of microbes is relatively high or stable in soil treated by alkaline fertilizers. It has been shown that the control of pH can be an effective means of disease control and maintaining a favorable soil ecosystem [29, 34].

In general, the analysis of AWCD by carbon source (Fig.2a) and the PCA plots of the present study revealed that the utilization of 2-hydroxybenzoic acid (phenols) by soil microbes in FOC-cultivated soil was remarkably higher than in soil with FOC inoculate. This result may be because FOC was the dominant microbe in inoculated soil, and FOC has a preference for phenols as carbon sources compared with other carbon sources. Moreover, it also revealed that the utilization of carbon sources (except pyruvic acid, methyl ester, D-Xylose, N-acetyl-D-glucosamine, and γ-hydroxybutyric acid) in soil treated with alkaline fertilizer was higher than in soils treated with conventional fertilizer. We conclude that the particular carbon sources favored by soil microbes, and their activity, can be influenced by the pH of the fertilizers applied, whether alkaline or conventional.

5. Conclusions

This study suggests that the activity and diversity of microbe communities in acid soil caused by conventional fertilizers is lowered by the presence of FOC inoculate, but is improved with the use of alkaline fertilizers. This study indicates that the management of soil pH through the application of alkaline fertilizers benefits the activity and diversity of microbes in acid soil. Alkaline fertilizer should be considered rather than conventional fertilizers to control soil acidification, especially in the banana fields seriously threatened by FOC.

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Table 2 Variance analysis of the diversity indices of soil microbial communities by fertilizer treatment *

	Inoculate	F1	F2	F3	F4	F5	Mean
AWCD	C1	0.947 ± 0.070	0.816 ± 0.082	0.752 ± 0.034	0.895 ± 0.031	0.968 ± 0.044	0.876 ± 0.068 [a]
	C2	0.652 ± 0.046	0.815 ± 0.024	0.695 ± 0.094	0.840 ± 0.029	0.705 ± 0.011	0.741 ± 0.061 [b]
	C3	0.720 ± 0.029	0.667 ± 0.034	0.724 ± 0.060	0.772 ± 0.102	0.946 ± 0.078	0.765 ± 0.080 [b]
	Mean	0.773 ± 0.089 [b, c]	0.766 ± 0.063 [b, c]	0.724 ± 0.060 [c]	0.836 ± 0.063 [a, b]	0.871 ± 0.085 [a]	—
S	C1	28.67 ± 0.67	26.00 ± 1.53	25.00 ± 1.16	27. 67 ± 1.45	28. 67 ± 0.67	27. 20 ± 1.31 [a]
	C2	23.00 ± 1.00	27.67 ± 0.33	24.33 ± 1.45	26. 33 ± 1.20	25. 00 ± 1.00	25. 27 ± 1. 2 [b]
	C3	24.00 ± 1.00	25.00 ± 2.08	23.67 ± 1.33	25. 33 ± 1.33	28. 67 ± 0.33	25. 33 ± 1.55 [b]
	Mean	25.22 ± 1.70 [b, c]	26. 22 ± 1.47 [a, b, c]	24. 33 ± 1.19 [c]	26. 44 ± 1.30 [a, b]	27. 44 ± 1. 23 [a]	—
D	C1	0.961 ± 0.001	0.957 ± 0.003	0.958 ± 0.002	0.959 ± 0.001	0.962 ± 0.001	0.959 ± 0.002 [a]
	C2	0.952 ± 0.002	0.957 ± 0.002	0.955 ± 0.003	0.956 ± 0.002	0.955 ± 0.001	0.955 ± 0.002 [b]
	C3	0.954 ± 0.001	0.955 ± 0.002	0.954 ± 0.002	0.957 ± 0.002	0.963 ± 0.001	0.956 ± 0.003 [b]
	Mean	0.956 ± 0.003 [b]	0.956 ± 0.002 [b]	0.956 ± 0.002 [b]	0.957 ± 0.002 [a, b]	0.960 ± 0.002 [a]	—
H'	C1	3.317 ± 0.009	3.222 ± 0.053	3.252 ± 0.038	3.278 ± 0.026	3.325 ± 0.018	3.279 ± 0.036 [a]
	C2	3.137 ± 0.042	3.247 ± 0.032	3.204 ± 0.041	3.220 ± 0.040	3.199 ± 0.020	3.201 ± 0.037 [b]
	C3	3.176 ± 0.024	3.202 ± 0.036	3.180 ± 0.051	3.230 ± 0.043	3.341 ± 0.011	3.226 ± 0.047 [b]
	Mean	3.210 ± 0.053 [b]	3.224 ± 0.038 [b]	3.212 ± 0.042 [b]	3.243 ± 0.036 [a, b]	3.288 ± 0.042 [a]	—

* The values are means ± standard error. Replicates are in triplicate.

Note: If the mean value of the data either in the same column or in the same line was followed by the same letter(s), it indicated that there is no significant difference among the data in the column or line.