# EFFECTS AND MECHANISMS OF ORGANIC ACID INPUT ON THE PRIMING EFFECT OF DARK BROWN SOIL

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**Abstract.** In this study, malonic acid was used to simulate the organic acids in the root exudates of *Fraxinus mandshurica* to study their impact of organic acids in root exudates on the priming effect mechanism of organic carbon. Through incubation experiments, $^{13}C$-labeled malonic acid was added to soil samples to study the effects of organic acids on the priming effect, physical and chemical properties, and bacterial diversity in dark brown soil. The results showed that after the addition of malonic acid, the cumulative soil respiration increased significantly, and soil organic matter generated a strong positive priming effect. There were no significant changes in the soil bacteria and microorganism species after adding malonic acid, but significant differences in species abundance were observed. Actinobacteria were dominant at the phylum level, followed by Firmicutes, Proteobacteria and Verrucomicrobia. At genus level, Bacillus, Micromonospora and Streptomyces were dominant during the whole incubation period. The addition of malonic acid demonstrated a strong priming effect on organic carbon present in this typical dark brown soil, showed relation with the soil physical and chemical properties, the typical microbial species, and the changes in the composition of the soil microbial community after the addition of fresh organic matter.
**Keywords:** *malonic acid, $^{13}C$-labeled, soil respiration, soil organic carbon, microbial diversity, priming mechanism*

## Introduction

The soil organic carbon pools play an important role in soil biology, with their size dynamically changing constantly, controlled by fixation and mineralization of plant organic carbon (Dai, 2018). Addition of exogenous substances to soil promotes the mineralization of organic carbon or organic nitrogen that is already present in the soil, and this is termed as the "priming effect" (Löhnis, 1926; Bingemann, 1953). It is necessary to properly understand the priming effect mechanism and its impact on mineralization, fixation, and equilibrium relationships in storing the forest soil organic matter, and the means to influence it to resolve agricultural production and global climatic problems.

The main exogenous substances in forest soils include forest floor litter and root exudates of forest plants, and the priming effect in forest soils is closely related to these. The root exudates are regarded as important carriers of material exchange and information transmission between plants and soil. They are the key factors in the micro-ecological rhizosphere characteristics of plants, and have important functions in biogeochemical cycles and in the regulation of soil micro-ecosystem structure and function (Woldendrop, 1963; Haichar, 2014), especially in the priming effect of soil organic carbon, regulation of complex interactions between soil and microorganisms,

and in the priming effect of soil organic carbon (Ezékiel et al., 2003). Therefore, many studies have been conducted on the priming effect of root exudates on soil organic carbon. There are studies that observed the positive effect played by the root exudates on priming effect, while few other studies have observed a negative role. For example, Helal and Sauerbeck (1984), Sauerbech and Sauerbeck (1986), Fu et al. (2002) have found that the root exudates of corn, sunflower and soybean had positive priming effects on soil organic carbon and promoted decomposition of soil organic matter; while Reid and Goss (2010) and Sparling et al. (2010) have found that the root exudates of wheat, barley and sorghum had a negative priming effect on soil organic carbon and inhibited the decomposition of soil organic matter. Moreover, some studies have reported that the effect of root exudates on soil priming effects depends on the specific component of exudates to be input. Among them, Dalenberg and Jager et al. (1989) have found that aspartic acid generated a positive priming effect, while glucose produced a negative one. Also, Landi et al. (2006) have found that oxalic acid produced a positive priming effect on soil organic carbon, while glucose showed no obvious effect. In contrast, Shen and Bartha (1996) have found that benzoic acid had no effect on soil organic carbon, but glucose enhanced it.

The results of these studies with regard to the priming effect of root exudates on soil are different, and the mechanism of priming process still remained unclear. It has been suggested that priming might be related to the type and quantity of root exudates and soil properties (Wu et al., 1993; Luna-Guido et al., 2003; Xie et al., 2005). Furthermore, it also might be related to the interactions between root exudates and soil microorganisms. According to Landi et al. (2006) addition of artificial root exudates has significantly affected the soil bacterial community. Fountaine et al. (2004) have proposed that microbial populations can be divided into R-strategy species and K-strategy species, and that competition between R- and K-strategists affects the strength of the priming effect. There are currently two explanations for the priming effect mechanism: the co-metabolic mechanism and the N mineralization mechanism. When the nutrient elements in the soil are sufficient, then the soil microbial population is dominated by r-strategy species, thus generating a positive priming effect through co-metabolic mechanisms. When nutrient elements in the soil are scarce, then the soil microbial population is dominated by K strategy species, thus generating the positive priming effect through N mining mechanism. However, it is unclear as to which of these mechanisms are triggered by root exudates. In addition, as root exudates are mixed, it is difficult to analyze the priming effect, and is impossible to determine as to which substance plays a major role. Therefore, when studying the soil priming effect of root exudates, a single organic compound should be applied to study the resulting mechanism. In addition, most of the studies use carbohydrate compounds for analyzing the effect of root exudates on soil priming mechanism, while the study of organic acids on soil priming are still lacking. What impact does the addition of organic acids would bring to soil organic carbon, and what specific changes would microbial community have the impact is still unknown.

Hence, in this study, the typical soil from the mid-temperate region of China was incubated, and used malonic acid to simulate organic acids. The interactions between organic acids in root exudates, and the mechanisms by which they affected the priming of soil organic carbon, and the effect of root exudates on soil bacterial diversity were examined. The main objectives of this study were: (1) to determine the effect of organic

acid on soil priming effect; and (2) to investigate how organic acid input change the soil properties and soil bacteria communities.

## Materials and methods

### Test soil

The soil test samples were collected from the Maoershan Experimental Forest Farm of MAPERSHAN, Harbin, China (45°20'N,127°30'E). The samples of dark brown soil were collected from the shallow layer at 0–10 cm depth using the snake shaped point sampling method. The soil samples were then taken to the laboratory for mixing and air drying, and the plant residues and other solid matter were removed as far as possible by screening with a 2 mm sieve. The basic physical and chemical properties of the soil were as follows: pH $6.7 \pm 0.05$; organic carbon content $11.12 \pm 11.95$ g/kg; total N content $1.42$ g/kg; $NH_4$-N content $396.19 \pm 3.43$ mg/kg; $NO_3$-N content $90.70 \pm 3.03$; $NO_2$-N content $0.27 \pm 0.01$ mg/kg; and available P content $40.02 \pm 1.01$ mg/kg.

### $^{13}C$ tracer experimental incubation method

In this study, $^{13}C$-labeled malonic acid was used to simulate the organic acids. This was determined as the greatest amount of organic acids in the root exudates of *Fraxinus mandshurica* in our previous study, and is regarded as the most important economic tree species in mid-temperate region of China (Li, 2019). A fresh organic matter (FOM) treatment group and a control group were established, with 3 repetitions in each group. Three FOM treatment subgroups of Mal-1, Mal-2 and Mal-3 were created, and a base of malonic acid with 3% soil organic carbon and $^{13}C$ marker were added into each, followed by 1%, 2% and 3% additional soil organic carbon, respectively, with 3 repetitions each. Next, 50 g of soil was weighed for each repetition and put into a 500 mL incubation flask with 20 mL of deionized water, the flask was sealed, and then was pre-incubated at 25 °C for 7 d. The whole process was performed in the dark. After pre-incubation, the water content in the incubation bottles was adjusted to $60 \pm 0.5\%$ distilled water and mixed evenly. At the same time, the same amount of distilled water was added to the control group and mixed evenly. A 25 mL beaker containing 15 mL 0.5 mol/L NaOH solution was carefully placed into each incubation flask, and the flasks were resealed and incubated at a constant temperature of $25 \pm 1$ °C to stimulate the unplanted soil in natural conditions. The beakers were then removed on days 1, 3, 7, 14, 21, 2, 42, and 56, and the NaOH solution in the beaker was collected and placed into a centrifuge tube. Fresh NaOH solution was added to the beaker, and incubated further (Wang et al., 2013; Qin et al., 2016). The levels of $CO_2$-C and $\delta^{13}C$ isotope (products of soil respiration) were determined for each of the NaOH solution samples. The $CO_2$-C was determined as total organic content (TOC), and $\delta^{13}C$ was determined by using an isotope mass spectrometer (Flash, 2000 HT, Thermo Scientific, Inc., Waltham, Massachusetts, U.S.). The results were used to analyze the effects of soil $CO_2$ emission and the resulting soil priming effect.

### Calculation of soil $CO_2$-C emission and the priming effect

1. Soil cumulative $CO_2$-C (Ctot) emissions: the sum total of the cumulative $CO_2$-C emissions of each sample.

2. Contribution of malonic acid and soil organic matter (SOM) to $CO_2$ emissions.

The total $CO_2$ ($C_{SOC}$) from SOM plus the total $CO_2$ ($C_{mal}$) from FOM were calculated according to the *Equations 1* and *2* (Qiu et al., 2019).

$$C_{SOC} = (\delta_{mal} - \delta_T)/(\delta_{mal} - \delta_C) \times C_T \qquad \text{(Eq.1)}$$

$$C_{mal} = C_T - C_{SOC} \qquad \text{(Eq.2)}$$

where $C_T$ represents the total C emission after adding FOM; $C_{SOC}$ represents the C emission from the soil organic carbon (SOC) source after adding FOM; $C_{mal}$ represents the C emission from the FOM source after adding FOM; $\delta_T$ represents the $\delta^{13}C$ of $CO_2$ after adding FOM; $\delta_C$ represents the $\delta^{13}C$ of $CO_2$ from control; and $\delta_{mal}$ represents the $\delta^{13}C$ of FOM.

### Calculation of the cumulative priming effect on the soil

Based on the determination of soil $CO_2$-C release and $CO_2\delta^{13}C$, the cumulative priming effect (PE) was calculated using *Equation 3* (Hamer and Marschner, 2005).

$$PE = C_{SOC} - C_C \qquad \text{(Eq.3)}$$

where $C_{SOC}$ represents C emission from SOC source after adding FOM and $C_C$ represents C emission from SOC in control.

### Calculation of the daily cumulative priming effect on the soil

The calculation of the daily priming effect on soil and daily mineralization rate of FOM was based on *Equations 4* and *5*.

$$PEt_{1\text{-daily}} = (PEt_2 - PEt_1)/(t2 - t1) \qquad \text{(Eq.4)}$$

$$FOMt_{1\text{-daily}} = (FOMt_2 - FOMt_1)/t2 - t1 \qquad \text{(Eq.5)}$$

where: t1 is the date of the sampling day; t2 is the date of the next sampling; PEt1 and PEt2 are the priming doses calculated during the sampling of t1 and t2, respectively; and $PEt_{1\text{-daily}}$ is the priming rate of t1. $FOM_{t1}$ and $FOM_{t2}$ are calculated as malonic acid mineralization amounts on days t1 and t2, respectively. $FOM_{t1\text{-daily}}$ is the malonic acid mineralization rate on day t1.

### Incubation method for the destructive sampling experiment

The group without incubation and without the addition of malonic marked was regarded as blank group. The analytically pure malonic acid was not added under consistent incubation conditions, and destructive samples were taken on days 3, 14, and 56, respectively (the three groups were marked as ck-1, ckl-2 and ck-3, respectively). The analytically pure malonic acid was added under consistent incubation conditions consistent using the $^{13}C$ tracer method. Destructive samples were taken on days 3, 14, and 56, respectively (the treatment groups are marked as mal-1, mal-2 and mal-3, respectively), and stored in a refrigerator at -80 °C for determining soil physical and chemical properties, and microbial diversity.

## Determination of soil physical and chemical indexes

The pH of the soil was determined using potentiometer (pH Meter Basic 20, Crison Instruments, Alella, Spain). The total C and total N contents of the soil were determined using an element analyzer (Vario EL, Analysensysteme GmbH, Hanau, Germany). The mineral N, which was determined as $NH_4$-N, $NO_3$-N and $NO_2$-N, were extracted using the KCl solution extraction method followed by determination with a flow analytical system (Smartchem 200, Zeal Quest Equipments, France) (Feng, 2019). The effective P in the soil was extracted using the double acid extraction method and a flow analytical system (Xu et al., 2014).

## Determination of the soil bacteria and microorganism community structure and diversity

### (1) Total DNA extraction and PCR amplification

The American FastDNA® Spin Kit was used to extract the total DNA in the soil samples. The concentration and purity of the extracted DNA were measured using a NanoDrop2000 Microvolume UV-Vis Spectrophotometer. The quality of the DNA was measured using 1% agarose gel electrophoresis. The V3-V4 variable region was amplified by PCR through 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. The amplification procedure was as follows: pre-denaturation at 95 °C for 4 min, 27 cycles; denaturation at 95 °C for 30 s; annealing at 55 °C; extension at 72°C for 30 s; and finally, extension at 72 °C for 10 min (using a PCR analyser: ABI GeneAmp® 9700). The amplification system was 20 uL, 4 uL 5 * FastPfu buffer, 2 uL 2.5 mm dNTPs, 0.8 uL primer (5 μm), 0.4 μL FastPfu polymerase, and 10 ng DNA template (Wijaya et al., 2019).

### (2) Identification, purification and quantification of polymerase chain reaction (PCR) products

Each sample was replicated with three PCR repeats and mixed with three repeated PCR products. Two percent agarose gel electrophoresis was used to detect the PCR products. The PCR products were then purified using an AxyPrep DNA Gel Extraction Kit. The PCR products were used for quantitative detection by using a Quantus™ Fluorometer. The corresponding proportions were mixed according to the sequencing quantity requirements of each sample (Belgrader, 1999).

### (3) Construction of a priming effect (PE) library and Illumina sequencing

A NEXTFLEX® Rapid DNA-Seq Kit was used to build the library and connect the adaptors. Magnetic beads were used to screen and remove the self-linked segments of the adaptors. PCR amplification was used to enrich the library template. Magnetic beads were used to recover the PCR products to obtain the final library. The sequence was carried out using a Miseq PE300 platform (Illumina company, Shanghai Major Biomedical Technology Co., Ltd.).

### (4) Analysis method

Trimmatotic software was used for quality control of the original sequencing, and Flash software was used for fragment assembly. UPARSE software (version 7.1 http://drive5.com/uparse/) was used to obtain operational taxonomic units (OTUs) by

clustering the sequences to a 97% similarity. A ribosomal database project (RDP) classifier (http://rdp.cme.msu.edu/) was used for species classification and annotation of each sequence. The Silva database (SSU128) was used for alignment by setting the alignment threshold to 70%. In addition, OTU analysis, Alpha diversity analysis, dilution curve analysis, Venn diagram analysis, bar diagrams and heat map analysis of colony compositions, and principal co-ordinates analysis (PcoA) analyses were performed. Species difference analysis of multi-group comparisons between groups and phylogenetic tree analyses were also performed.

### Statistical treatment

The experiment was conducted three times parallelly. SAS software was used for one-way ANOVA and Duncan analysis, and SigmaPlot 12.5 was used to prepare the figures and diagrams.

## Results and analysis

### Effects of malonic acid addition on soil respiration, and priming effects

After addition of malonic acid, the soil cumulative respiration has reached to 3831.52 mg C/kg at the end of incubation. However, the respiration of control soil has reached only to 204.63 mg C/kg instead (*Fig. 1*). The priming effect induced by mal was obvious, and the cumulative priming during the whole incubation period was 2874.69 mg/kg. As shown in *Figure 2,* the priming effect of soil caused by malonic acid was decreased with increasing incubation time. The priming effect remained the strongest on day 1 of incubation, then decreased rapidly in intensity, reaching the lowest level after 21 days, and then showed a slight upward trend. The C released in soil respiration of malonic acid mineralization was 546.23 mg/kg, in which only 4.55% that of the added amount. As shown in *Figure 3,* the mineralization rate of malonic acid was decreased with increasing incubation time. It remained the strongest on day 2 of incubation and then its intensity was decreased rapidly. After 7 days of incubation, the mineralization effect was weak. The accumulated mineralization effect due to malonic acid accounted for 95.65% of the total mineralization during the first 7 days of incubation.

### Effects of malonic acid addition on soil physical and chemical properties

As shown in *Table 1,* the pH levels showed no significant differences between the treatments, and so no effect of added malonic acid was observed on soil pH. As shown by the TOC measurements in the three treatment groups, blank, the control group (ck1) and the experimental group Mal-1, no significant differences were observed in TOC between blank and ck1, while both were significantly lower than that of Mal-1 treatment after malonic acid addition. The soil TOC values were gradually decreased with increasing incubation time, and the treatments differed significantly ($p < 0.05$). Soil organic C was rapidly decomposed during the early stage of incubation, and the decomposition rate was slowed down during the middle and late stages of incubation. The $NH_4$-N, $NO_3$-N, and mineral N results showed that all the three were increased with increasing incubation time, and showed significant changes. In addition, $NH_4$-N and $NO_3$-N content in the groups without adding malonic acid were significantly higher than in the corresponding groups. The results showed no significant differences in the available phosphorous (AP) among the treatment groups.
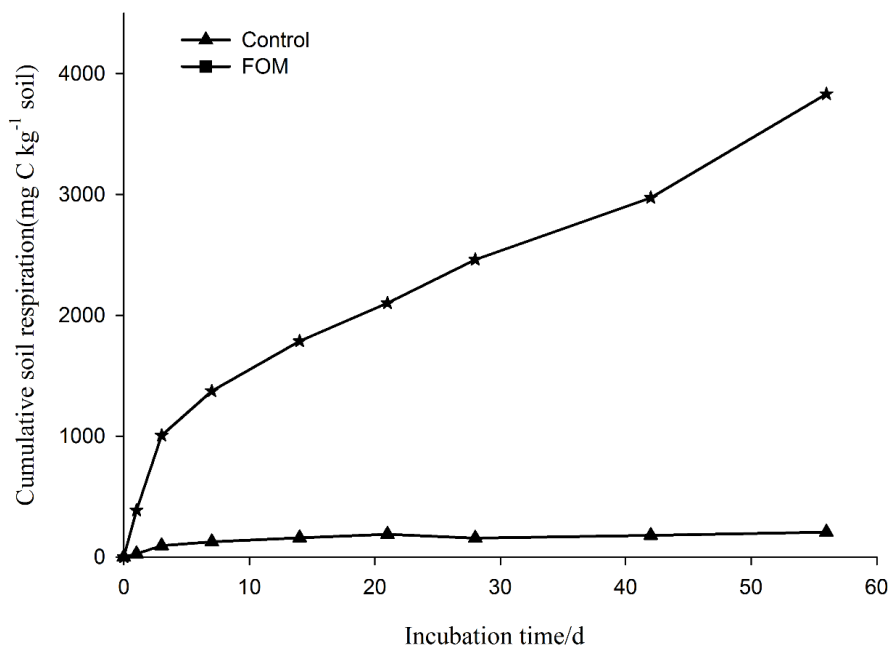
*Figure 1.* The dynamics of cumulative $CO_2$-C emission derived from different carbon sources (n = 3). FOM: malonic acid addition was 3%. Control: no added malonic acid
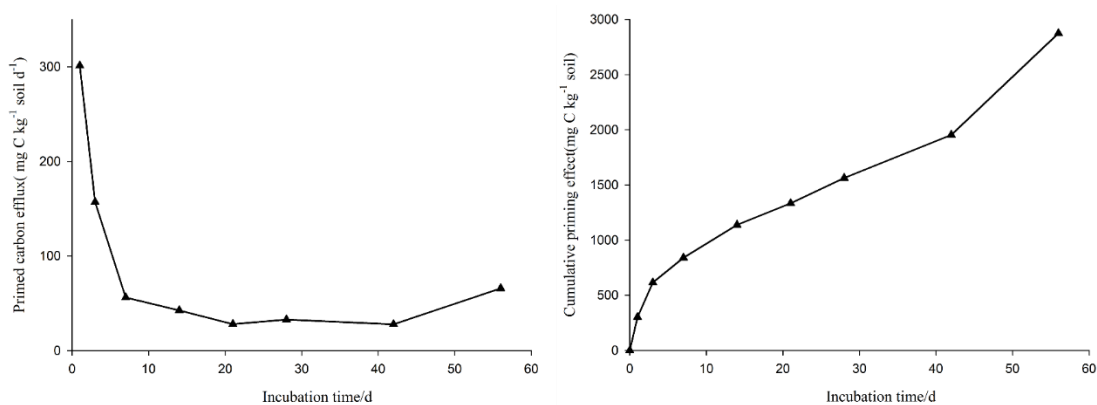


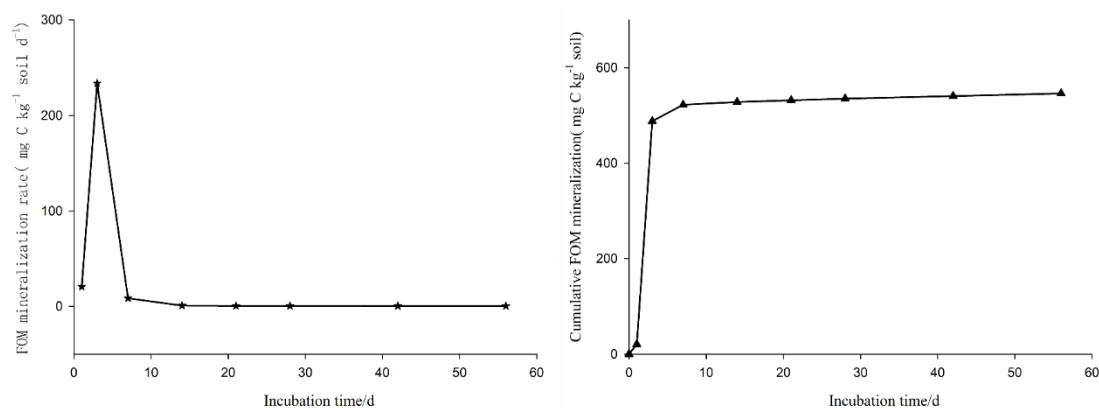*Figure 2.* The priming effects and cumulative priming effects of the soil (n = 3)



*Figure 3.* The mineralization effects and the cumulative mineralization effects of the soil (n = 3)

***Table 1.*** *the influence of malonic acid addition on soil physicochemical properties (n = 3)*

| No. | pH | TOC mg/kg | NH₄-N mg/kg | NO₃-N mg/kg | Total inorganic N g/kg | AP mg/kg |
|---|---|---|---|---|---|---|
| blank | 6.70 ± 0.05 [a] | 832.48 ± 11.95[b] | 396.19 ± 3.43[f] | 90.70 ± 3.03[f] | 486.89 ± 3.36[f] | 40.02 ± 1.01[a] |
| ck1 | 6.68 ± 0.02[a] | 866.97 ± 40.17[b] | 539.25 ± 6.10[d] | 115.54 ± 2.12[cd] | 654.79 ± 4.27[d] | 42.82 ± 1.21[a] |
| ck2 | 6.68 ± 0.02[a] | 588.69 ± 38.56[d] | 662.11 ± 26.82[b] | 125.95 ± 4.89[c] | 788.06 ± 16.75[b] | 43.90 ± 0.78[a] |
| ck3 | 6.67 ± 0.02[a] | 538.14 ± 15.64[e] | 756.70 ± 25.00[a] | 150.56 ± 1.70[a] | 907.26 ± 18.34[a] | 41.77 ± 1.69[a] |
| Mal-1 | 6.67 ± 0.03[a] | 942.39 ± 46.79[a] | 476.60 ± 12.32[e] | 106.22 ± 4.82[e] | 582.82 ± 9.58[e] | 39.67 ± 1.05[a] |
| Mal-2 | 6.65 ± 0.01[a] | 673.67 ± 24.26[c] | 581.16 ± 11.22[c] | 116.57 ± 5.42[c] | 697.73 ± 8.31[c] | 41.54 ± 1.03[a] |
| Mal-3 | 6.67 ± 0.02[a] | 598.35 ± 12.91[d] | 739.63 ± 12.62[a] | 143.32 ± 5.29[b] | 882.95 ± 10.57[a] | 42.24 ± 1.13[a] |

a-d show intra-group differences (p < 0.05). TOC: total organic carbon; AP: available phosphorous; Blank: The group without incubation and without the addition of malonic acid; ck-1: the group culture for 3 days without malonic acid; ck-2: the group culture for 14 days without malonic acid; ck-3: the group culture for 56 days with malonic acid. Mal-1: the group culture for 3 days with malonic acid; Mal-2: the group culture for 14 days with Malonic acid; Mal-3: the group culture for 56 days with malonic acid

## Effects of malonic acid addition on soil bacterial diversity

Analyzing the OTU dilution curve and species accumulation curve at 97% similarity level revealed that both curves were relatively flat in all the treatment groups, showing that the amount of sequencing data used was adequate and that the depth of sample measurement was reliable. The sample size and library capacity were clearly large enough to represent that the majority of the bacteria in the experimental soil flora, and the species richness and library diversity have reached saturation point.

## Diversity analysis of Venn graphs and PCoA

Venn graphs were generated to count the number of common and unique species in the samples, and to assess the similarity and overlap between the species composition in different samples. As shown in *Figure 4,* 1385, 1475 and 1449 OTUs were detected in Mal-1, Mal-2 and Mal-3 groups, respectively. Of the total 964 OTUs, Mal-1 and Mal-2 shared 137 OTUs; Mal-1 and Mal-3 shared 124 OTUs; and Mal-2 and Mal-3 shared 201 OTUs. Mal-1, Mal-2, and Mal-3 had 160, 173, and 160 unique OTUs, respectively, which accounted for 11.55%, 11.43%, and 11.04% of the total OTUs. The results showed that the number of bacterial species were increased initially and then were decreased with increasing incubation time, and showed significant differences in the composition of soil bacterial communities during different incubation periods. Principle component analysis (PCA) was carried out on the abundance of OTUs in each sample using 97% similarity level as the principle variable. The principal component factors were extracted from 16S rRNA sequences of each sample, and a PCA map was drawn using R language tools. As shown in *Figure 5,* the first two principal components, PC1 and PC2, accounted for 60.68% and 16.48% variation, respectively. Their cumulative contribution was 77.16%, and most of the variation was explained. *Figure 5* shows that the distribution distances between the experimental groups and the blank group was large, and significant. It can also be seen that the distribution distances of the experimental groups with different incubation times were relatively large, and significantly different, indicating that the structure of the bacterial flora of each

experimental group had significant differences. The distance between Mal-2 and Mal-3 was relatively close, and the distance between Mal-2 and Mal-3 was relatively far from ck and Mal-1 in the control group. This indicated that the bacterial flora of Mal-2 and Mal-3 samples was relatively similar, and that the difference between ck and Mal-1 samples showed more significance. However, the distance between ck and Mal-1 was relatively close, indicating that the bacterial flora of ck and Mal-1 were relatively similar. Overall, the results showed that the structure of the soil bacterial communities were significantly different at different incubation times. The structure of the bacterial communities were similar during the middle and end of the incubation period, while there were large differences between them at the end of incubation.
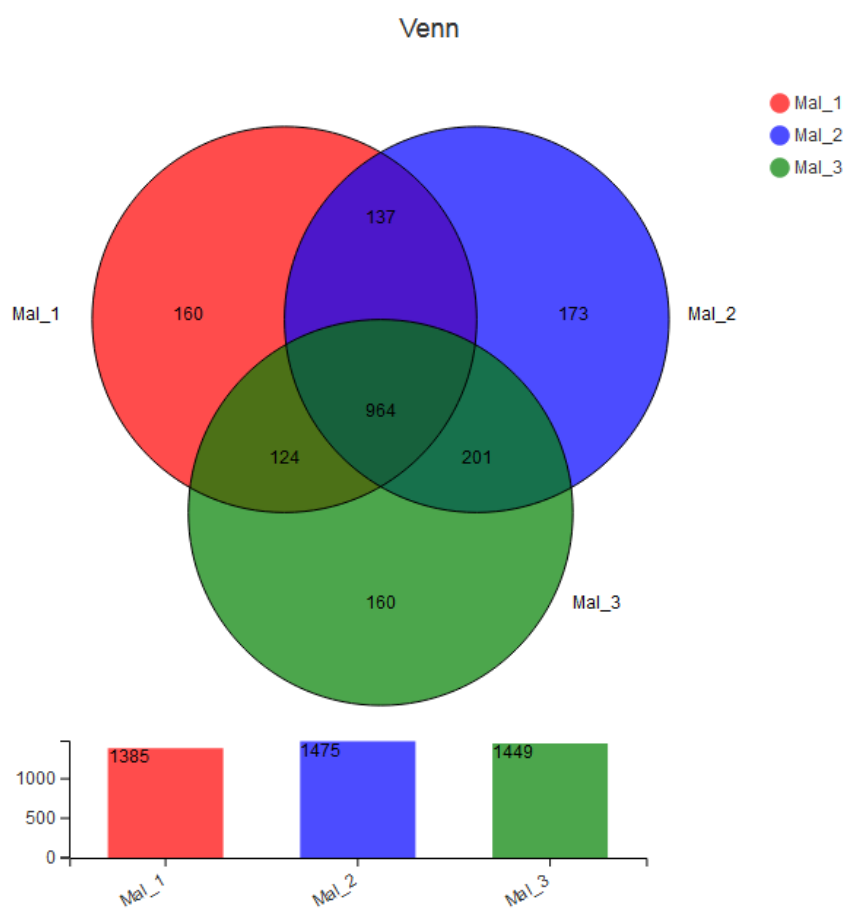


*Figure 4. A Venn diagram analysis of OTUs (97% sequence similarity). Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; and Mal-3: this group was incubated for 56 days with malonic acid*

### Soil bacterial community composition analysis of the samples

*(1) Community diversity of soil bacteria at the phylum level*

The dominant bacteria of each group were as follows (*Fig. 6a; Table 2*): Actinobacteria were shown to be dominant, followed by Firmicutes and Proteobacteria. The structure within each group was similar, but the proportions of the three groups differed. Actinomycetes were increased significantly during the early stage of incubation, Proteobacteria were increased significantly during the middle stage of

incubation, and Actinomycetes were increased significantly again in the later stage of incubation when some bacteria in other phyla also showed an increasing trend.
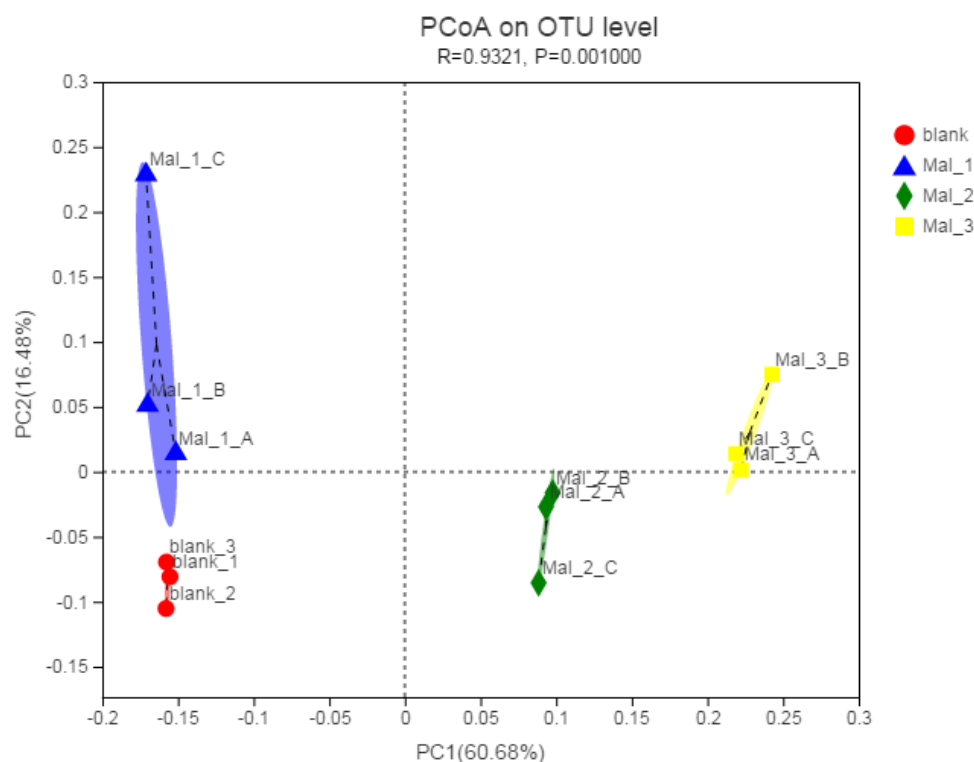


***Figure 5.*** *The principal coordinates analysis (PCoA) analysis of OTUs (97% sequence similarity). PC1 and PC2: principal component1 and principal component2; Blank: The group without incubation and without the addition of malonic acid; Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid; A, B and C: parallel sample*

***Table 2.*** *The bacterial composition at phylum level*

| Proportion of phylum (%) | Group | | | |
|---|---|---|---|---|
| | **Blank** | **Mal-1** | **Mal-2** | **Mal-3** |
| Actinobacteria | 44.70% | 57.37% | 39.82% | 47.92% |
| Firmicutes | 23.76% | 23.72% | 24.11% | 13.56% |
| Proteobacteria | 14.53% | 10.13% | 23.75% | 24.45% |
| Verracomicrobia | 8.38% | 4.02% | 4.12% | 4.44% |
| Chloroflexi | 3.78% | 2.22% | 2.12% | 2.20% |
| Acidobacteria | 2.51% | 1.21% | 1.41% | 1.26% |
| Bacteroidetes | 0.60% | 0.22% | 2.58% | 2.16% |
| Gemmatimonadetes | 0.7% | 0.46% | 0.83% | 2.22% |
| Planctomycetes | 0.37% | 0.14% | 0.68% | 1.41% |
| Others | 0.67% | 0.51% | 0.58% | 0.38% |
| Total | 100% | 100% | 100% | 100% |

Blank: The group without incubation and without the addition of malonic acid; Mal-1: the group culture for 3 days with Malonic acid; Mal-2: the group culture for 14 days with malonic acid; Mal-3: the group culture for 56 days with malonic acid

*(2) Community diversity of soil bacteria at the genus level*

The *Bacillus* species were dominated (*Fig. 6b; Table 3*), followed by the *Micromonospora* and *Streptomyces* species. The species structure of each group was similar, but the proportions of bacterial genera differed. The number of *Micromonospora* spp. was increased significantly during the early stage of incubation, while that of *Bacillus* spp. was increased significantly during the middle stage of incubation. In addition, the similarity between the bacterial species during the middle and end stages of incubation was higher than that between the control group and the initial stage of incubation, which was consistent with the results of the PCoA analysis.
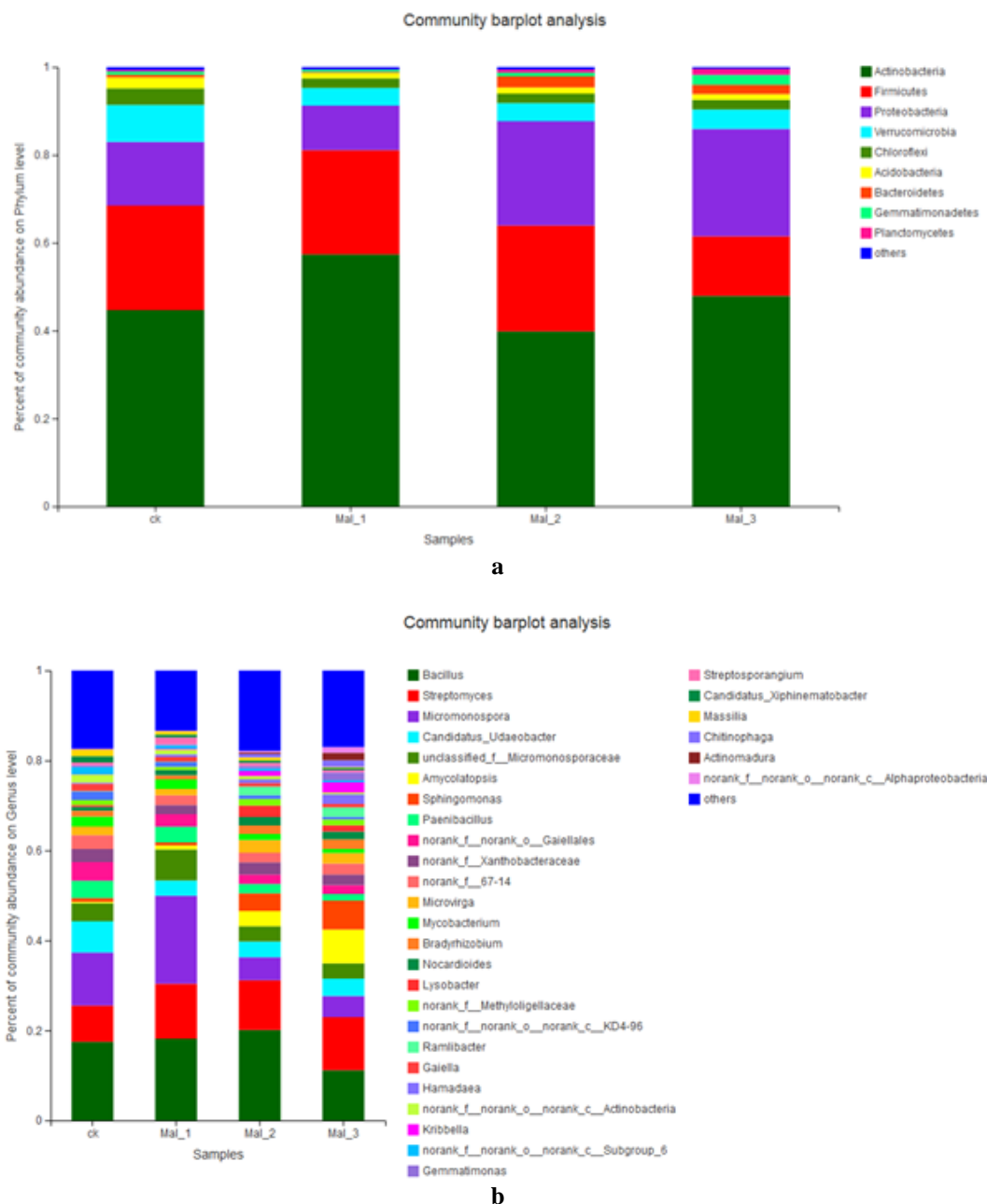


a



b

***Figure 6.*** *Bar community composition analysis at (a) phylum and (b) genus levels. Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid*

***Table 3.*** *The bacterial composition at genus level*

| Proportion of genus (%) | Group | | | |
|---|---|---|---|---|
| | **Blank** | **Mal-1** | **Mal-2** | **Mal-3** |
| Bacillus | 17.49% | 18.18% | 20.11% | 11.15% |
| Micromonospora | 11.83% | 19.61% | 5.10% | 4.69% |
| Streptomyces | 8.01% | 12.18% | 11.05% | 11.83% |
| Unclassified_f_Micromonosporaceae | 4.06% | 6.92% | 3.40% | 3.41% |
| Candidatus_Udaeobacter | 6.91% | 3.36% | 3.49% | 3.85% |
| paenibacillus | 3.87% | 3.49% | 2.11% | -- |
| norank_f_norank_o_Gaiellales | 4.08% | 2.77% | 2.06% | -- |
| norank_f_67-14 | 3.10% | 2.17% | 2.14% | 2.48% |
| norank_f_xanthobacteraceae | 2.92% | 2.02% | 2.72% | 2.37% |
| Microvirga | 1.90% | 1.46% | 2.82% | 2.30% |
| Mycobacterium | 2.24% | 2.05% | -- | -- |
| Gaiella | 1.60% | -- | -- | -- |
| norank_f_norank_o_ norank_c_KD4-96 | 1.97% | 1.12% | -- | -- |
| norank_f_norank_o_orank_c_Actinbaterria | 1.72% | | | |
| Streptosporangium | -- | 1.55% | -- | -- |
| Bradyrhizobium | -- | -- | -- | 2.12% |
| Nocardioides | -- | 1.15% | -- | -- |
| Hamadaea | -- | -- | -- | 2.12% |
| Sphingomonas | -- | -- | 3.89% | 6.40% |
| Amycolatopsis | -- | -- | 3.42% | 7.5% |
| Lyspbacter | -- | -- | 2.60% | -- |
| Ramlibacter | -- | -- | 1.93% | -- |
| Kribbella | -- | -- | -- | 2.28% |
| Others | 28.3% | 21.97% | 33.16% | 40.91% |
| Total | 100% | 100% | 100% | 100% |

Blank: The group without incubation and without the addition of malonic acid; Mal-1: the group culture for 3 days with malonic acid; Mal-2: the group culture for 14 days with malonic acid; Mal-3: the group culture for 56 days with malonic acid

## *Sample species difference analysis*

Different significance tests and analyses between species groups were carried out for samples at different incubation times. At the phylum level, the abundance of Actinobacteria, Proteobacteria, Bacteroidetes, Nitrospirae, Gemmatimonadetes, Planctomycetes, and Entotheonellaeota showed significant differences among the four groups (one-way ANOVA, $p < 0.05$) (*Fig. 7a*). At the genus level, Micromonospora spp., Micromonosporaceae spp., Amycolatopsis spp., Sphingomonas spp., Paenibacillus spp., unidentified_Order_Gaiellales, unidentified_Family_Xanthobacteraceae, unidentified_Family_67-14, Microvirga spp., Mycobacterium spp., Bradyrhizobium spp., Nocardioides spp., Lysobacter spp., unidentified_Family_Methyloligellaceae, unidentified_Family_O, and unidentified_C_KD4-96 showed significant or extremely significant differences among the four treatment groups (one-way ANOVA, $p < 0.05$) (*Fig. 7b*).
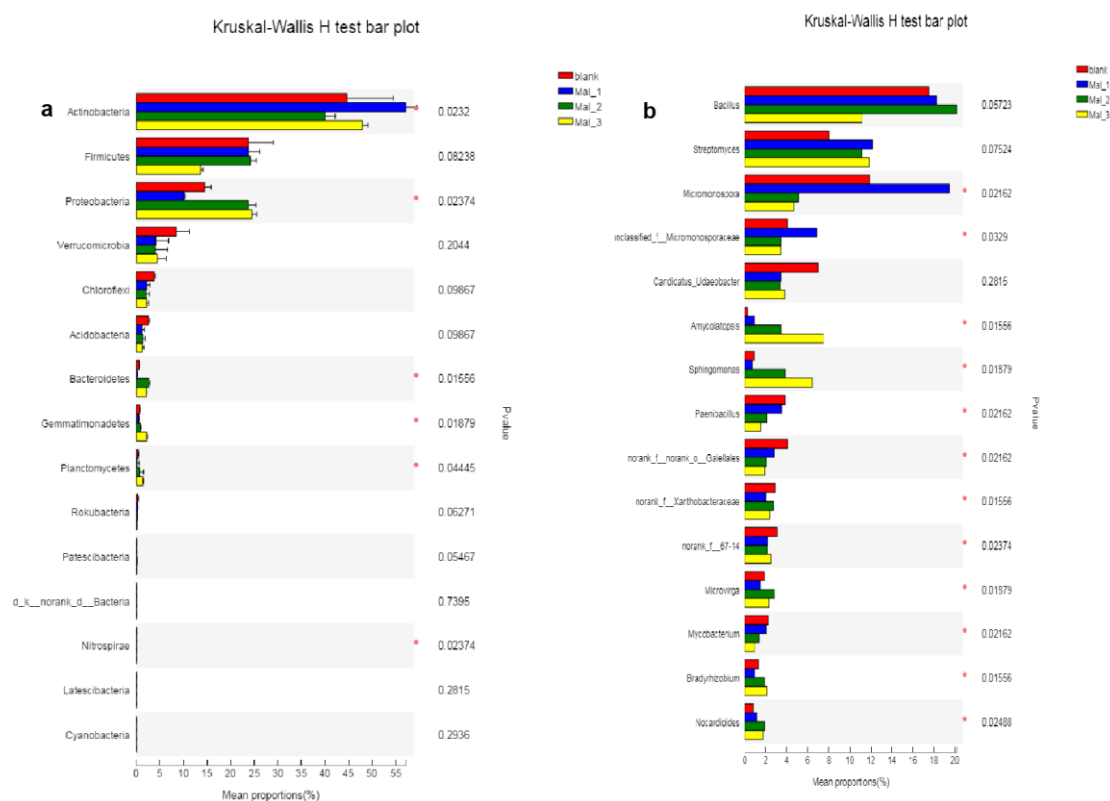
***Figure 7.*** *The analysis of differences between species composition at a, phylum and b, genus levels. Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid*

## Discussion

### *Effects of malonic acid addition on soil carbon mineralization and the priming effects*

Following the addition of malonic acid, the cumulative respiration of the soil has been increased due to malonic acid mineralization by soil microorganisms, causing SOM to generate a strong positive priming effect. Previous studies have also shown that addition of organic acids into soil can promote mineralization of the original SOM, resulting in positive priming effect (Lonardo et al., 2017). A strong positive priming effect was found after adding oxalic acid to soil (Dutton et al., 1996), and this is possibly because oxalic acid can help fungal basidiomycetes to depolymerize cellulose and lignocellulose. In this study, the priming effect caused by the addition of malonic acid was shown to be positive during the whole incubation period, and remained the highest during the early stage of incubation. This might be due to the fact that during the early stage of incubation, just after the application of FOM to the soil, the dissolved organic carbon (DOC) content remained high and had sufficient energy available, and this led microorganisms to generate strong positive priming effects through co-metabolism while mineralizing the organic acids (Blagodatskaya et al., 2014). As incubation time increases, the DOC content in the soil is gradually decreased, resulting in decreased priming effect (Ma et al., 2018). However, the mineralization peak of malonic acid also occurred during the early stage of incubation, on day 3, before decreasing rapidly. The mineralization rate showed no significant fluctuation thereafter

until the end of the incubation period. The accumulated malonic acid mineralization was only 4.55% that of the amount added initially, which accounted for relatively low values reported in other studies (Yuan et al., 2015; Wang et al., 2016). This might be because the FOM applied in this study was low molecular weight organic matter, and the substrate utilization efficiency of low molecular weight matter remained very high because microorganisms required no increase in extra-cellular enzyme metabolism during the process of decomposition and did not required not many enzymatic reactions. This in turn reduced the respiratory metabolism of microorganisms, so that their substrate utilization efficiency was low (Bosatta, 1999; Blagodatskaya and Kuzyakov, 2008; Öquist et al., 2017). At the same time, the availability of nutrient elements in the soil also affected their substrate availability. When the nutrient content was high, then the organic matter utilization capacity of microorganisms and the substrate availability were increased (Ågren et al., 2001; Manzoni et al., 2012). Additionally, the total N and P contents of the soil used in this study were very high, in which the N content was about 40 times higher and the P content was about 30 times higher than in other studies (Yuan et al., 2015; Wang et al., 2016). The mineralization rate of malonic acid in this study was therefore low.

### Effects of malonic acid addition on the physical and chemical properties of the soil

Experiments on root soil have revealed that the application of organic acids can affect soil pH, thus increasing the availability of nutrients in the soil (Neumann and Römheld, 1999; Jones et al., 2010). In particular, it can affect the P content of the soil and increase the AP levels (Gardner et al., 1983; Marschner et al., 1986). However, in this study there were no significant changes in soil pH and AP after adding malonic acid. But the mineral N content in the soil was increased significantly with increasing incubation period after adding malonic acid, which is possibly due to the release of the mineral N by soil microorganisms while mineralizing SOM (Fontaine et al., 2004). Moreover, the mineral N content of the soil after malonic acid addition was less than that in the control group throughout the incubation period, and this is possibly due to the fact that soil microorganisms consume large amounts of existing mineral N when decomposing additional SOM and malonic acid. At the same time, the DOC content of the soil was increased significantly at the beginning of incubation period after adding malonic acid, and then decreased with increasing incubation time. The addition of FOM has quickly increased the DOC content of the soil, however, the mineralization of available substrate in the soil was increased with increasing incubation time, resulting in the decrease of available substrate in the soil and decrease of DOC content (Mary et al.,1992; Jans-Hammermeister, 1998).

### Effects of added malonic acid on soil bacterial community structure

After addition of malonic acid, Actinobacteria were the dominant population, followed by Firmicutes, Proteobacteria and Verrucomicrobia. Actinobacteria were the most abundant during the early stage of incubation, but were decreased significantly during the middle stage of incubation, before rising to a certain extent during the later stage. After Actinobacteria, the population of *Micromonospora* spp. was significantly increased during the early stage of incubation, while *Streptomyces* spp. showed no significant differences in their abundance. Previous studies have also shown that Actinobacteria are dominant during the early stage of incubation (Padmanabhan et al.,

2003), and this is perhaps because they tend to decompose organic matter more easily and can decompose cellulose at the same time. Firmicutes was the second most abundant microbial population during the early stage of incubation, but showed no significant differences with that during the middle and late stages of incubation. *Bacillus* spp. (Firmicutes) were the most abundant of the entire microflora throughout the entire incubation period, but did not differ significantly in abundance between the early, middle and late stages of incubation. As typical r-strategists, Firmicutes genera form the first breeding pioneer populations after the addition of FOM into the soil. Studies have shown that extracellular enzymes secreted by Firmicutes can decompose FOM and SOM at the same time (Fierer et al., 2007; Pascault et al., 2013). In addition, Chloroflexi and Verrucomicrobia are also r-strategists (Razanamalala, 2018), but no significant difference were observed in their abundance throughout the entire incubation period. We speculate that the priming effect observed might be caused by the co-metabolism of Actinobacteria dominated by *Micromonospora* spp. and bacteria dominated by *Bacillus* spp.

Proteobacteria are a well-known r-strategy group (Padmanabhan et al., 2003; Fierer et al., 2007), and ranked third in abundance throughout the entire incubation process, although their abundance during the early stage of incubation was significantly lower than that during the middle and late stages of incubation. This indicated that this r-strategy group was dominant during the middle and late stages of incubation, and that the positive priming effects were caused by co-metabolism. A study found that the priming effects during the later stage of incubation were related to Acidobacteria and Planctomycetes spp. (Razanamalala, 2018). In this study, the highest abundance of Acidobacteria spp. occurred during the early stage of incubation, while the population abundance of Planctomycetes and Gemmatimonadetes spp. increased significantly with increased incubation time. Previous studies have shown that these three phyla are K-strategists (Fierer et al., 2007; DeBruyn et al., 2011; Pascault et al., 2013). Marilley and Aragno (1999) have found that the abundance of Acidobacteria in rhizosphere soil is lower than that in non-rhizosphere soil (Marilley and Aragno, 1999). In addition, some studies showed that the abundance of Acidobacteria is lower in soil with more available energy (McCaig et al., 1999; Axelrood et al., 2002). However, in our study the highest abundance of Acidobacteria occurred at the end of the pre-incubation period, and was decreased after addition of malonic acid. This showed that when the available energy in the soil increases, the r-strategists in the soil increase and propagate vigorously, resulting in the loss of competitive advantage of K-strategists and their population decline (Héry et al., 2005). However, some studies have shown that Gemmatimonadetes can mineralize the mineral N by using some FOM or easily decomposed SOM in soil as an energy source (Razanamalala,). Therefore, in this study, we speculated that during the later stage of incubation, when energy and nutrient reserves in the soil became gradually consumed, the competitive advantage of K-strategists (Planctomycetes and Gemmatimonadetes) in the soil was relatively enhanced (Fontaine et al., 2004). This caused a priming effect through N mining mechanism, wherein the mineral N content during the later stage of incubation was significantly higher than during the early stage of incubation. Therefore, we speculate that the later priming effects were caused by the co-metabolism of r-strategy species, which are dominated by Proteobacteria, while the positive stimulation of K-strategy species, which are dominated by Gemmatimonadetes and Planctomycetes, resulted due to increased N availability.

## Conclusion

The addition of malonic acid significantly increased $CO_2$ respiration in dark brown soil. The SOM had a positive priming effect, but the cumulative mineralization of malonic acid remained low. The addition of malonic acid did not affect the soil pH and effective P, but increased the mineral N content of the soil. The addition of malonic acid had no significant effect on various species of soil bacteria and microorganisms present, but the relative abundance of each species was significantly affected with incubation time. In conclusion, addition of malonic acid had a strong priming effect on the original organic C content of dark brown soil, which was not only related to the physical and chemical composition of the soil, but also to the microbial populations in the soil and the changes in soil microbial community composition. Future studies should focus on the effect of malonic acid addition on the diversity of soil fungi, and the interactions between bacteria and fungi in order to further reveal the mechanisms behind the priming effects of organic acids in root exudates on soil organic C.

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