International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:6, No:12, 2012

Numbers and Biomass of Bacteria and Fungi Obtained by the Direct Microscopic Count Method

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***Abstract*—**The soil ecology of the organic and mineral soil layers of laurel-leaved and *Cryptomeria japonica* forest in the Kasuga-yama Hill Primeval Forest (Nara, Japan) was assessed. The number of bacteria obtained by the dilution plate count method was less than 0.05% of those counted by the direct microscopic count. We therefore found that forest soil contains large numbers of non-culturable bacteria compared with agricultural soils. The numbers of bacteria and fungi obtained by both the dilution plate count and the direct microscopic count were larger in the deeper horizons (F and H) of the organic layer than in the mineral soil layer. This suggests that active microbial metabolism takes place in the organic layer. The numbers of bacteria and the length of fungal hyphae obtained by the direct count method were greater in the H horizon than in the F horizon. The direct microscopic count revealed numerous non-culturable bacteria and fungi in the soil. The ratio of fungal to bacterial biomass was lower in the laurel-leaved forest soil. The fungal biomass was therefore relatively low in the laurel-leaved forest soil due to differences in forest vegetation.

***Keywords*—**Bacterial number, Dilution plate count, Direct microscopic count, Forest soil.

I. Introduction

THE world heritage Kasuga-yama Hill Primeval Forest in Nara, Japan, is a lowland laurel-leaved forest, in which natural conditions have been preserved for more than 1160 years [1]. As numerous unique species of plants, animals, and insects have been identified in this forest, it has attracted considerable research attention [1]. However, the microbial ecology of the soil supporting this primeval forest has not been well studied.

In forest ecology, research is often focused on the microbial processes affecting the decomposition of soil organic matter, which are influenced by differences in vegetation, climate, and soil type [2]. Although soil microorganisms are typically quantified using the dilution plate count method, this standard method only detects approximately 1% of the total number of bacteria present in soil [3]. The accuracy of fungal counts are also limited using methods that only count spores suited to specific culture medium [4]. In the present study, the numbers and biomass of bacteria and fungi present in organic and mineral soil layers from laurel-leaved and *Cryptomeria japonica* forests were examined and compared using the dilution plate and direct microscopic count methods.

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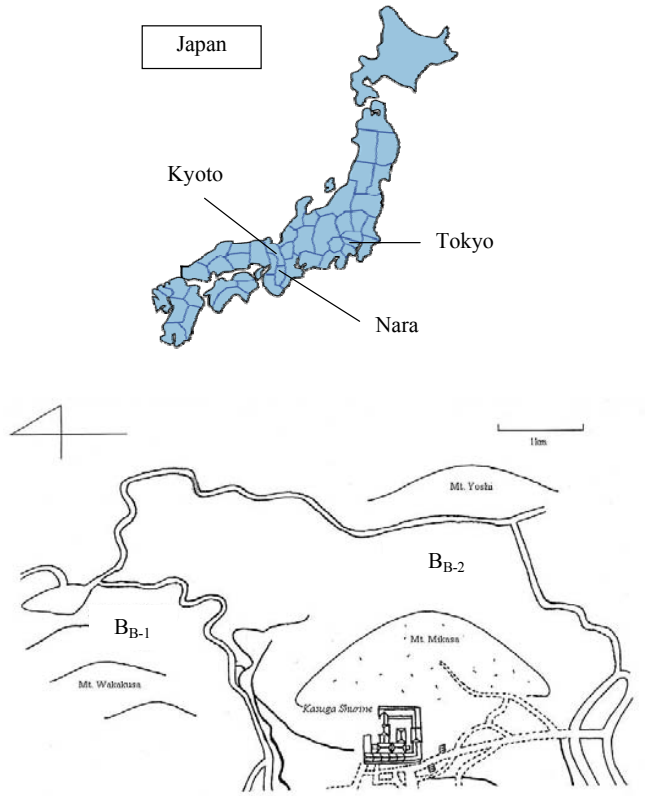

Fig. 1 Kasuga-yama Hill Primeval Forest in Nara, Japan. Soil samples were obtained from the laurel-leaved and *C. japonica* forests, as indicated

II. Materials and Methods

A. Soil Samples

An outline of the Kasuga-yama Hill Primeval forest in Nara, Japan is illustrated in Fig. 1. Soil samples were collected from the laurel-leaved (BB-1) and *C. japonica* forests (BB-2), whose predominant vegetation is *Machilus thunbergii* and *Neolitsea aciculata*, and *C. japonica*, respectively, in April 2001. All collected samples were dry brown forest soils. The soil was divided into five layers, consisting of organic (L, F, and H

horizons) and mineral soil (A and B horizons) layers (Fig. 2). Cross-sectional observation of each layer was performed to identify the most medial point between layers, from which several sub-samples were collected. Samples were placed in polyethylene bags and preserved at lower than 4 °C until the day of analysis. Samples were cultured in an incubator (MIR-153, SANYO) overnight at 25 °C prior to analysis. For microbial analyses, the particle sizes of the soil samples from each horizon were adjusted to <2 mm using sterilized scissors.

For soil chemical analyses, soil samples with <2 mm particles were further adjusted to <0.5 mm with a grinder (WB-1, Osaka Chemical Co. Ltd.). Soil samples were analyzed for pH, and moisture, carbon (C), and nitrogen (N) contents (Table I).

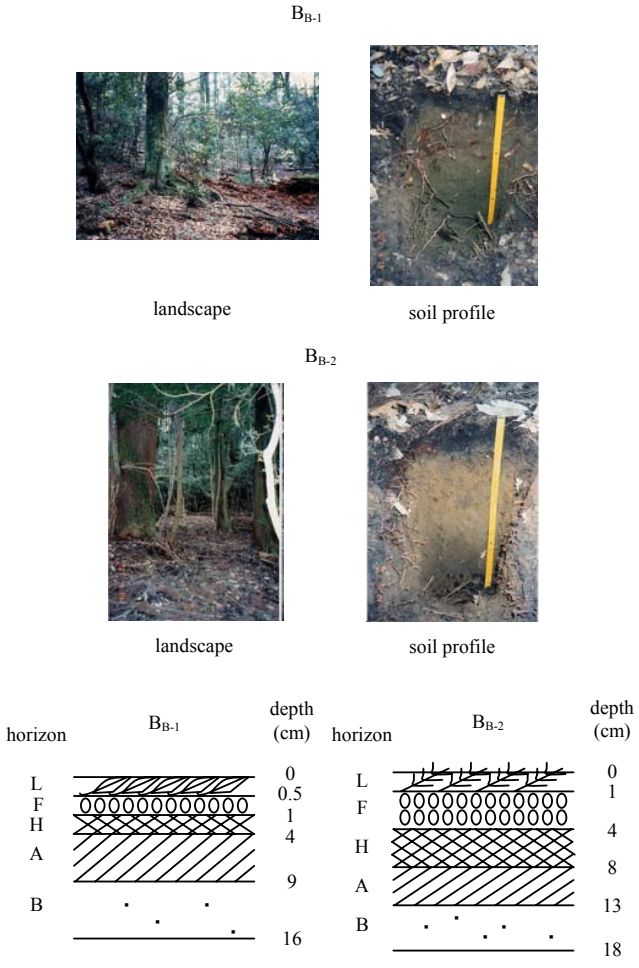

Fig. 2 Soil profiles of laurel-leaved and *C. japonica* forests

B. Measurements of Soil Microorganisms

Bacteria and fungi in the soil samples were quantified using two standard methods: the dilution plate count and direct microscopic count methods.

Size-adjusted soil samples (5 g) were suspended in 45 mL bacterial removed sterile water in blender cups and then homogenized for 3 min at 12,000 rpm with a Homo blender (500C, Sakuma) [5].

For the dilution count method, Albumin and Rose Bengal-Streptomycin agar were used to culture bacteria and fungi contained in sample solutions at 25 °C for 4 or 6 d [4].

The direct microscopic count method for quantifying bacteria was performed according to the method of Someya [6]. Briefly, sample solutions were dispersed using an ultrasonic device (US-3, Kenis) for 20 min, diluted ten fold in bacterial removed sterile water, and 100 µL of the resulting solution was then filtered through a 0.2 µm filter. A 50 µL ethidium bromide solution (100 µg mL–1) was then placed onto the filter for 3 min, and was then filtered. The filter was dried, placed onto a glass slide, and bacteria were counted under a fluorescence microscope (FX-35A, Nikon) under oil immersion.

Fungal hyphae length was measured according to the Jones-Mollison method [7]. Briefly, sample solutions were dispersed using an ultrasonic device (US-3, Kenis) for 5 min, and 1 mL of resulting solution was added to 9 mL of 1.5% agar solution, and an agar membrane of the resulting mixture was placed on a haemocytometer. The membrane was stained with phenol-aniline blue solution for 1 h, and was then de-stained with 97% ethanol. The length of stained fungal hyphae was measured under a light microscope (TK, Kagaku Kyoeisha).

The soil bacterial biomass (*Bm*) and fungal biomass (*Fm*) were calculated using the following formulae [8]:

$$Bm\ (\text{mg g–1}) = n \times vave \times \rho \times r \quad (1)$$

$$Fm\ (\text{mg g–1}) = v \times \rho \times r \quad (2)$$

where n = numbers of bacteria (cells g–1); *vave* = average bacterial volume (= 0.19 µm3); ρ = specific gravity of microorganisms (= 1.1 g cm–3); r = rate of dry matter (= 20%); and v = fungal volume (cm3 g–1).

TABLE I
PROPERTIES OF LAUREL-LEAVED AND *C.JAPONICA* FOREST SOILS

Vegetation	Horizon	Moisture Content (%)	pH (H2O)	Total C (g kg–1)	Total N (g kg–1)	C/N ratio	Volume weight (g cm–3)
BB-1	L	13.7	4.52	467.3	12.6	37.1	0.048
	F	43.8	4.34	402.2	16.9	23.8	0.180
	H	56.5	4.51	318.9	16.9	18.9	0.297
	A	42.5	3.88	189.1	10.5	18.0	0.685
	B	25.0	3.64	38.2	2.0	19.1	0.823
BB-2	L	13.7	5.04	498.7	5.8	89.1	0.032
	F	62.0	4.37	361.2	11.9	30.4	0.066
	H	53.4	3.87	201.4	9.5	21.2	0.155
	A	42.0	3.81	106.0	5.8	18.3	0.493
	B	28.4	3.95	30.7	2.0	15.4	0.628

III. RESULTS

A. Dilution Plate Counts of Forest Soil Bacteria and Fungi

In both the laurel-leaved and *C. japonica* forest soils, the numbers of bacteria and fungi quantified using the dilution plate count method (Fig. 3) were larger in the organic layer (F and H horizons) than in the mineral soil layer. A comparison of the three organic layer horizons revealed that the numbers of

bacteria and fungi were lowest in the L horizon and highest in the F horizon.

Bacterial and fungal dilution plate counts in the laurel-leaved organic layers were higher than those in the *C. japonica* organic layers. In contrast, the mineral soil layers of *C. japonica* forest were found to contain more microbes than those of laurel-leaved forest.

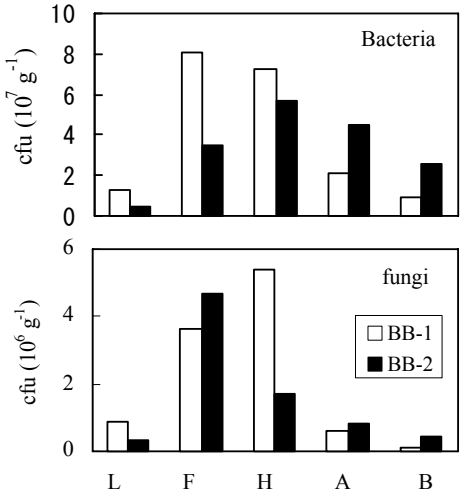

Fig. 3 The numbers of bacteria and fungi in laurel-leaved (BB-1) and *C. japonica* (BB-2) forest soils obtained by the dilution plate count method

B. Direct Microscopic Counts of Soil Bacteria

Using the direct microscopic count method, a significantly larger number of bacteria were detected (Fig. 4) than that obtained by the dilution plate method, which only measures culturable soil bacteria. The numbers of bacteria obtained by the two methods showed similar trends: more bacteria were present in the F and H horizons of the organic layer than in the mineral soil layer, and the bacterial count was also greater in the laurel-leaved forest soil compared with the *C. japonica* forest soil. However, the layer distribution of bacteria assessed using the direct plate count method did not necessarily correspond to the results from the dilution plate count method. Specifically, the numbers of bacteria obtained by the direct count method were greater in the H horizon than in the F horizon, while the laurel-leaved forest bacteria in the A horizon of the mineral soil layer were found to be present at the identical level in the F and H horizons of the organic layer.

In agricultural and grassland soils, the concentration of bacteria determined using the direct microscopic count method has been reported to range from 3.5-6.6×109 and 1.0-19.7×109 g-1, respectively [9]. Here, the concentration of bacteria in the A horizon of forest soil, which is most comparable to agricultural soils, was 9.3-42.1 × 109 g-1. Notably, the bacterial concentration *C. japonica* forest soil was similar to grassland soils, whereas that in laurel-leaved forest soil more closely resembled agricultural soils. Moreover, the number of bacteria in the F and H horizons of the organic layer greatly exceeded that typically found in agricultural soils.

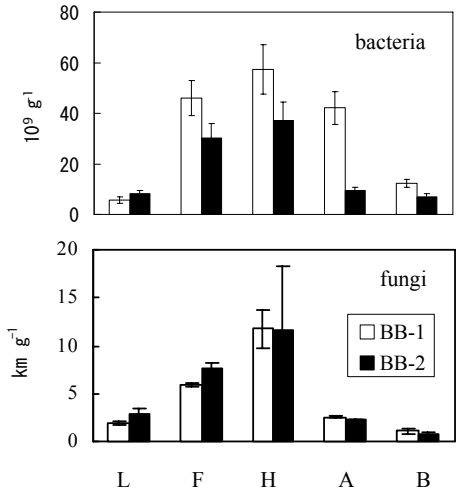

Fig. 4 The bacterial number and fungal hyphae length in laurel-leaved (BB-1) and *C. japonica* (BB-2) forest soils determined by the direct microscopic count method

Table II shows the ratio of the bacterial count values obtained using the dilution plate count (P) and direct microscopic count methods (D) for each horizon of the laurel-leaved and *C. japonica* forest soils. The P/D of the A horizon forest soil ranged from 0.05%-0.48%. Interestingly, these rations were markedly lower than the reported P/D value of 0.73%-3.1% for agricultural soils [9].

TABLE II
RATIO OF BACTERIAL NUMBER OBTAINED BY THE DILUTION PLATE COUNT (P) AND DIRECT MICROSCOPIC COUNT (D) METHODS (P/D)

Horizon	BB-1	BB-2
L	0.24	0.06
F	0.17	0.12
H	0.13	0.15
A	0.05	0.48
B	0.08	0.37

C. Fungal Hyphal Length Measured using the Direct Microscopic Count Method

Fungal hyphal length, as measured by the direct microscopic count method (Fig. 4), was longer in the deeper horizons (F and H) of the organic soil layer than that in both horizons of the mineral soil layer. Notably, the length of fungal hyphae was greater in the H horizon than in the F horizon. When each horizon was compared, fungal hyphae were found to be longest in the H horizon.

The length of fungal hyphae obtained by direct microscopic measurement for agricultural and grassland soils was previously reported to range from 1.03-1.79 and 0.04-2.30 km g-1, respectively [9]. Here, the fungal hyphal length of the A horizon forest soil was 2.22-2.50 km g-1, which was similar to that found in grassland soils. However, the length of fungal hyphae in the F and H horizons of the organic layer was significantly longer than that of typical agricultural soils.

D. Bacterial and Fungal Biomass

We also quantified the amount of bacterial (*Bm*) and fungal (*Fm*) biomass in the laurel-leaved and *C. japonica* forest soil [consider if it should be briefly described how it was quantified] (Table III). The total microbial biomass (*Bm* + *Fm*) in the organic layer was significantly greater than that detected in the mineral soil layer. For both forest soils, the total microbial biomass in the organic layer increased with soil depth, and was greatest in the H horizon. In contrast, the total soil biomass in the mineral soil layer decreased with increasing depth. The laurel-leaved forest soil was found to contain higher total biomass (*Bm* + *Fm*) than that of the *C. japonica* soil. Although the total soil biomass of the forest soil A horizons was similar to those previously reported for agricultural soils (1.6-3.0 mg g–1) [9], overall, the forest soil organic layers were far richer in microorganisms than agricultural soils.

The ratio of bacterial to fungal biomass (*Fm*/*Bm*) was the highest in the H horizon of the organic layer for both forest types. In addition, the total *Fm*/*Bm* in the laurel-leaved forest soil was less than that in the *C. japonica* soil, likely due to vegetation effects. The *Fm*/*Bm* of agricultural soils typically ranges 0.98-3.75 [10], which is similar to the *Fm*/*Bm* of A horizons in the forest soils observed here. The organic layer H horizons of the forest soil exhibited markedly higher *Fm*/*Bm* compared with agricultural soils.

TABLE III
BACTERIAL (*BM*) AND FUNGAL (*FM*) BIOMASS IN LAUREL-LEAVED AND *C. JAPONICA FOREST* SOILS

Horizon	BB-1			
	Bm (mg g–1)	*Fm* (mg g–1)	*Bm* + *Fm* (mg g–1)	*Fm*/*Bm*
L	0.23	0.75	0.98	3.21
F	1.93	5.96	7.89	3.09
H	2.40	18.51	20.91	7.71
A	1.76	1.97	3.73	1.12
B	0.51	1.46	1.97	2.84

Horizon	BB-2			
	Bm (mg g–1)	*Fm* (mg g–1)	*Bm* + *Fm* (mg g–1)	*Fm*/*Bm*
L	0.35	1.35	1.70	3.87
F	1.27	4.32	5.58	3.41
H	1.55	17.04	18.60	10.96
A	0.39	1.25	1.64	3.20
B	0.29	0.32	0.61	1.13

IV. DISCUSSION

Our examination of Kasuga-yama Hill Primeval Forest soils revealed that the numbers of bacteria and fungi obtained by both dilution plate count and direct microscopic count methods were larger in the deeper horizons (F and H) of organic layers than in mineral soil layers. This finding suggests that active microbial metabolism preferentially occurs in organic soil layers. However, the soil layer distribution of bacteria and fungal hyphae assessed using the two quantification methods did not necessarily correspond, as the values obtained by the direct count method were greater in the H horizon than in the F horizon. Differences in the distribution of soil microorganisms were clearly reflected in the bacterial biomass detected in each examined layer. The uppermost L horizon, which contains large amounts of fresh plant residue, contained low levels of microorganisms. Increased microbial biomass was detected in the adjacent F horizon, as nutrient levels are higher due to the advanced state of plant decomposition in this layer, while in the H horizon, which contains completely decayed organic matter, the amount of bacteria able to be grown in nutrient-poor conditions increased, relative to the culturable bacteria.

The bacterial numbers in the A of horizon forest soil, which is most comparable to agricultural soils, quantified using the dilution plate count method was similar to those reported for agricultural soils. However, the use of the direct microscopic count method resulted in a significantly higher estimated number of bacterial in the A horizon than in agricultural soils. Therefore, it appears that Kasuga-yama Hill Primeval Forest soil contains large numbers of non-culturable bacteria compared with agricultural soils. As this finding suggests the high complexity of microbial metabolism in forest soil, further research to identity and characterize the functions of these bacteria is necessary to better understand the microbial ecology of forest soil.

The total bacterial and fungal biomass of the forest soil A horizons was similar to those previously reported for agricultural soils. However, the forest organic layers overall were far richer in microorganisms than agricultural soils. Forest soil is a natural ecosystem that is continuously supplied with plant matter, and differs from agricultural soils, in which organic matter is disturbed by cultivation and readily consumed by abundant nutrient-starved crops.

Interestingly, the laurel-leaved forest soil had greater total bacterial and fungal biomass than the *C. japonica* forest soil. This difference was clearly due to differences in forest vegetation, which appeared to influence several salient soil properties, particularly the pH, and content of C and N in the H horizon. From the viewpoint that microbial biomass serves as reserve nutrients of plants, increased microbial biomass in the laurel-leaved forest indicates a higher metabolic activity than that of the *C. japonica* forest soil.

A comparison of the ratio of fungal to bacterial biomass revealed that the laurel-leaved forest soil had a lower ration than *C. japonica* soil, a finding that was also likely due to differences in forest vegetation. Notably, the H horizons of both forest soil organic layers had a much greater ratio of bacterial to fungal biomass compared with that previously reported for agricultural soils. However, the ratios in the forest soil A horizons were similar to that reported for agricultural soils. In addition, although the mineral soil layer of forest soils is generally considered to contain a greater proportion of fungal biomass than agricultural soils, our results show that the fungal content of the mineral soil layers of Kasuga-yama Hill Primeval Forest is similar to that of agricultural soil.

V. CONCLUSION

The microbial ecology of the organic and mineral soil layers of the laurel-leaved and *C. japonica* forests in Kasuga-yama Hill Primeval Forest (Nara, Japan) was found to be influenced by forest vegetation. The direct microscopic count method

revealed that significantly more non-culturable bacteria and fungi are present in the forest soil compared with typical agricultural soil.

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