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# Comparative Analysis of Soil Enzyme Activities between Laurel-Leaved and *Cryptomeria japonica* Forests

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***Abstract*—**Soil enzyme activities in Kasuga-yama Hill Primeval Forest (Nara, Japan) were examined to determine levels of mineralization and metabolism. Samples were selected from the soil surrounding laurel-leaved ($B_{B-1}$) and *Carpinus japonica* ($B_{B-2}$ and $P_w$) trees for analysis. Cellulase, β-xylosidase, and protease activities were higher in $B_{B-1}$ samples those in $B_{B-2}$ samples. These activity levels corresponded to the distribution of cellulose and hemicellulose in the soil horizons. Cellulase, β-xylosidase, and chymotrypsin activities were higher in soil from the $P_w$ forest than in that from the $B_{B-2}$ forest. The relationships between the soil enzymes calculated by Spearman's rank correlation indicate that the interactions between enzymes in $B_{B-2}$ samples were more complex than those in $P_w$ samples.

***Keywords*—**Comparative analysis, enzyme activities, forest soil, Spearman's rank correlation.

## I. Introduction

THE Kasuga-yama Hill Primeval Forest in Nara, Japan, is a World Heritage Site consisting of lowland laurel-leaved forest where natural conditions have been preserved for more than 1,000 years [1]. This primeval forest has attracted considerable attention due to the number of unique species of plants, animals, and insects living here that are found nowhere else on earth [1]. To our knowledge, however, the microbial ecology of the soil supporting this forest has not been studied in detail.

In forest ecology, microorganisms decompose organic soil matter from plant residue as a source of nourishment, which they then absorb. During this process, microorganisms produce enzymes that convert organic matter to carbon dioxide, water, ammonia, and other compounds [2], [3] (Fig. 1). The biochemical metabolism of microorganisms can therefore be indirectly investigated by measuring the activities of these enzymes.

Here, we applied comparative analysis to the activities of eight enzymes produced by microorganisms during the decomposition of organic matter in forest soil. Results demonstrated that interactions between enzymes might help characterize forest soil environments. To our knowledge, few studies have examined enzymes derived from different microorganisms that cooperatively decompose organic soil matter. These findings might therefore help clarify the mechanism by which organic soil matter is decomposed in the actual environment.

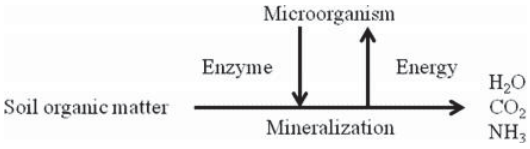


Fig. 1 Decomposition of soil organic matter by microorganisms

## II. Materials and Methods

### A. Soil Samples

Details regarding the Kasuga-yama Hill Primeval Forest in Nara, Japan are illustrated in Fig. 2. In April 2001, samples were collected from the soil surrounding laurel-leaved ($B_{B-1}$), *Carpinus japonica* ($B_{B-2}$), and Hideyoshi *C. japonica* trees ($P_w$), with predominant soil vegetation of *Machilus thunbergii* and *Neolitsea aciculata*, and *C. japonica*, respectively. $B_{B-1}$ and $B_{B-2}$ samples were dry, brown forest soils, and $P_w$ samples were wet podzol.

Soil samples were divided into four layers, consisting of organic (L, F, and H horizons) and mineral soil (A horizon) sub-layers (Fig. 3). Cross-sectional observation of each layer was conducted to identify the most medial point between layers, from which several sub-samples were collected. Samples were placed in polyethylene bags and preserved below 4 °C until the day of analysis. Prior to analysis, samples were cultured overnight in an incubator (MIR-153; Sanyo, Tokyo, Japan) at 30°C. For measurement of enzyme activities, the size of particles in the soil samples of each horizon was adjusted to <2 mm using sterilized scissors.

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

### B. Measurement of Soil Enzyme Activities

The enzyme activity of exocellulase, β-glucosidase, β-xylosidase, polyphenol oxidase, phosphomonoesterase, phosphodiesterase, chymotrypsin and trypsin in soil samples were measured using the methods described below. As these

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enzymes were isolated from microorganisms in the soil, their measurement was considered representative of the overall soil ecosystem in Kasuga-yama Hill Primeval Forest.

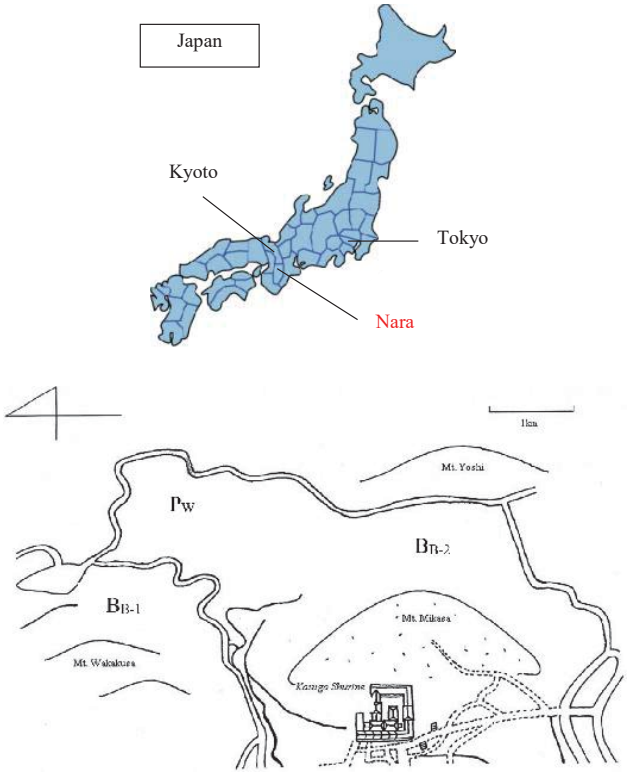


Fig. 2 Kasuga-yama Hill Primeval Forest in Nara, Japan: Soil samples were obtained from the laurel-leaved ($B_{B-1}$), *C. japonica* ($B_{B-2}$) and Hideyoshi *C. japonica* ($P_W$) forests, as indicated

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

| Vegetation | Horizon | Moisture Content (%) | pH ($H_2O$) | Total C (g $kg^{-1}$) | Total N (g $kg^{-1}$) | C/N ratio | Volume weight (g $cm^{-3}$) |
|---|---|---|---|---|---|---|---|
| $B_{B-1}$ | 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 |
| $B_{B-2}$ | 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 |
| $P_W$ | L | 19.3 | 4.95 | 505.0 | 7.4 | 68.2 | 0.033 |
| | F | 65.6 | 4.04 | 464.0 | 13.4 | 34.6 | 0.108 |
| | H | 68.0 | 3.95 | 269.0 | 12.3 | 21.9 | 0.176 |
| | A | 68.4 | 3.58 | 142.0 | 6.4 | 22.2 | 0.699 |

For exocellulase, β-glucosidase, β-xylosidase, phosphomonoesterase, and phosphodiesterase [4]-[7], a sample (L horizon: 0.2 g, F and H horizons: 0.5 g and A horizon: 1.0 g) of each culture was placed in a test tube and left to stand for 10 min with 0.2 mL toluene as a bacteriostatic agent. Samples were shaken (MMS; Eyela, Tokyo, Japan) for 1 h in an incubator (MIR-153; Sanyo Tokyo, Japan) at 30 °C with 2 mL of universal buffer and 1 mL of substrate solution corresponding to each enzyme (Table II). The enzyme reaction was then terminated by the addition of 8 mL of ethanol. Following centrifugation at 2,000 rpm for 5 min (Pasolina MD-15; Iuchi Tokyo, Japan), 0.5 mL of the supernatant was extracted and added to 2 mL of 50% ethanol. Absorbance at 400 nm was measured (UV-1200; Shimadzu, Kyoto, Japan) by performing color development with 1 mL of 1 M tris-aminomethane solution. Enzyme activities (n mol $g^{-1}$ $min^{-1}$) were measured five times and calculated according to the difference from the experimental value.

TABLE II
SUBSTRATE CORRESPONDING TO ENZYME

| Enzyme | Substrate |
|---|---|
| Exocellulase | p-Nitrophenyl-β-D-cellobioside |
| β-glucosidase | p-Nitrophenyl-β-D-glucopyranoside |
| β-xylosidase | p-Nitrophenyl-β-D-xylopyranoside |
| Polyphenoloxidase | Pyrocatechol |
| Phosphomonoesterase | p-Nitrophenylphosphate, Disodium Salt |
| Phosphodiesterase | Bis (p-Nitrophenyl) Hydrogenphosphate |
| Chymotrypsin | N-Benzoyl-L-argininamyde |
| Trypsin | z-Phenylaranylreusin |

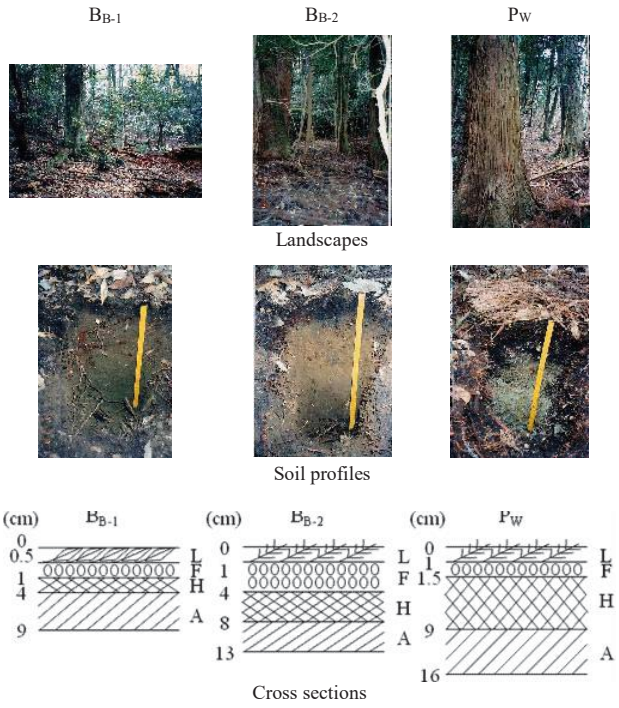


Fig. 3 Landscapes, soil profiles and cross sections of laurel-leaved, *C. japonica* and Hideyoshi *C. japonica* forests

For polyphenol oxidase [8], a sample (L horizon: 0.5 g, F, H, and A horizons: 1.0 g) of each culture was placed in a test tube. Samples were left for 2 min in a water bath at 30 °C with 2 mL of distilled water (DW), 1.2 mL of 1g $kg^{-1}$ L-ascorbic acid, and 2 mL of 20 mM pyrocatechol. The enzyme reaction was then terminated by the addition of 0.6 mL of 0.1 g $g^{-1}$ phosphoric acid. Filtrate was titrated to blue with 5 mM iodine solution. Enzyme

activities (n mol $g^{-1}$ $min^{-1}$) were measured three times and calculated according to the difference from the experimental value.

For chymotrypsin and trypsin [9], a sample (L horizon: 0.2 g, F, H, and A horizons: 0.5 g) of each culture was placed in a test tube and left to stand for 10 min with 0.2 mL toluene as a bacteriostatic agent. Samples were shaken (MMS, Eyela) for 1 h in an incubator (MIR-153, Sanyo) at 30 °C with 2 mL of 0.2 M phosphate buffer and 2 mL of substrate solution corresponding to each enzyme (Table II). The enzyme reaction was then terminated by the addition of 0.2 mL of 5 M hydrochloric acid. Following centrifugation at 2,000 rpm for 5 min (Pasolina MD-15, Iuchi), 0.5 mL of the supernatant was extracted to a test tube, and 1 mL of ninhydrin solution was added. This solution was then boiled for 15 min, and 6 mL of 50% ethanol was added. Absorbance at 570 nm was measured (UV-1200, Shimadzu). Enzyme activities (n mol $g^{-1}$ $min^{-1}$) were measured five times and calculated according to the difference from the experimental value.

### C. Statistical Methods

Spearman's rank correlation was performed to estimate the association between enzyme activities. This model infers an association due to the calculation of a partial correlation coefficient matrix from the correlation coefficient matrix, which is only possible when the latter is regular. On occasion, we therefore combined hierarchical clustering to remove redundant data from recorded enzyme activities.

The concept of conditional independence is fundamental to GGM. In this graph, each variable is represented by a vertex, and two vertices are connected by an edge if they share a direct association. In contrast, any pair of vertices not connected in the graph is conditionally independent. Conditional independence is estimated by the partial correlation coefficient, which is expressed using the following equation:

$$r_{ij} = 1 - \frac{6\sum_{l=1}^{N}(R_{il} - R_{jl})^2}{N^3 - N}$$

where $\boldsymbol{r_{ij}}$ denotes the Spearman's rank correlation between the soil enzyme $\boldsymbol{i}$ and $\boldsymbol{j}$, $\boldsymbol{R_{il}}$ denotes the rank number of enzyme $\boldsymbol{i}$ at case $\boldsymbol{l}$ (layer $\boldsymbol{l}$), and $\boldsymbol{N}$ denotes the total number of layers.

To evaluate which pairs of factors were conditionally independent, we applied the covariance selection attained using the stepwise and iterative algorithm developed by Wermuth and Scheidt [10]. When the partial correlation coefficient for a pair of factors is equal to 0, they are conditionally independent, and the relationship is expressed as the absence of an edge between the nodes corresponding to the clusters in the independence graph. In other words, the graph represents the enzyme activity network in each soil sample.

$$R_{ij} = \max F_{ij}$$

where $\boldsymbol{R_{ij}}$ denotes the decided activity rank of enzyme $\boldsymbol{i}$ at layer $j$ (L, F, H, A), and $\boldsymbol{F_{ij}}$ denotes the frequency of ordered numbers determined from the measured data.

## III. Results and Discussion

### A. Soil Enzyme Activities

Soil enzyme activities in each horizon are shown in Fig. 4.

Enzyme activities tended to be higher in organic layers than in mineral soil layers for all forest types, suggesting the presence of a large number of microorganisms that secrete external enzymes and decompose plant residue as a substrate (energy source).

Exocellulase, β-glucosidase, and β-xylosidase activities were higher in the L horizon than in others, but protease activities were higher in the deeper horizons (F and H horizons) of organic layers. The uppermost L horizon consists of high levels of fresh plant residue and microorganisms that decompose cellulose and hemicellulose. In the F horizon, the proportion of microorganisms that absorbed protein from plant nutrients increased as carbon decomposition progressed.

Comparison of enzyme activities of soil organic matter showed that $B_{B\text{-}1}$ samples had a faster metabolism than $B_{B\text{-}2}$ samples. This was also due to differences in forest vegetation, which appear to influence several salient soil properties—particularly the pH and C and N content in the F and H horizons. This result is consistent with the fact that $B_{B\text{-}1}$ soil generally contains a higher proportion of more decomposable organic matter, such as water-soluble polysaccharides, hemicellulose, cellulose, and protein, and a lower proportion of less decomposable organic matter, such as lipid and resin. Similarly, $P_W$ samples also had faster metabolism than $B_{B\text{-}2}$ samples. Although lipid, resin, and cellulose content were similar between the two locations, soil from $P_W$ contained more water-soluble polysaccharides, hemicellulose, and protein.

### B. Associations between Soil Enzymes

The inferred network between soil enzymes within each soil type is shown in Fig. 5.

In the organic layers (L, F and H horizons), carbon sources are more abundant in $B_{B\text{-}1}$ than $B_{B\text{-}1}$ type soil. Activation of C decomposing enzymes is considered to be co-operative.

The total mass of organism (C and N) is smallest in $B_{B\text{-}2}$ type soil, even though the organic layer is thicker than $B_{B\text{-}1}$. To make the organic layer from a few substances, many types of enzymes work together.

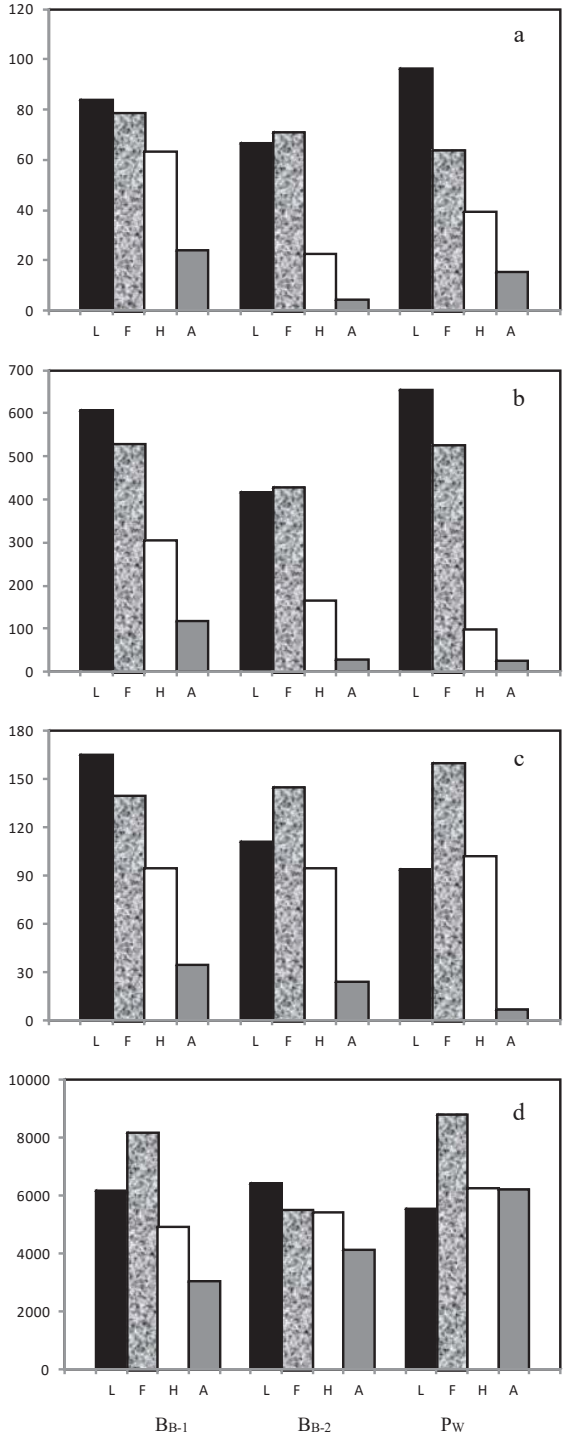


Fig. 4 (a) Soil enzyme activities in each horizon a: exocellulase, b: β-glucosidase, c: β-xylosidase, d: polyphenol oxidase

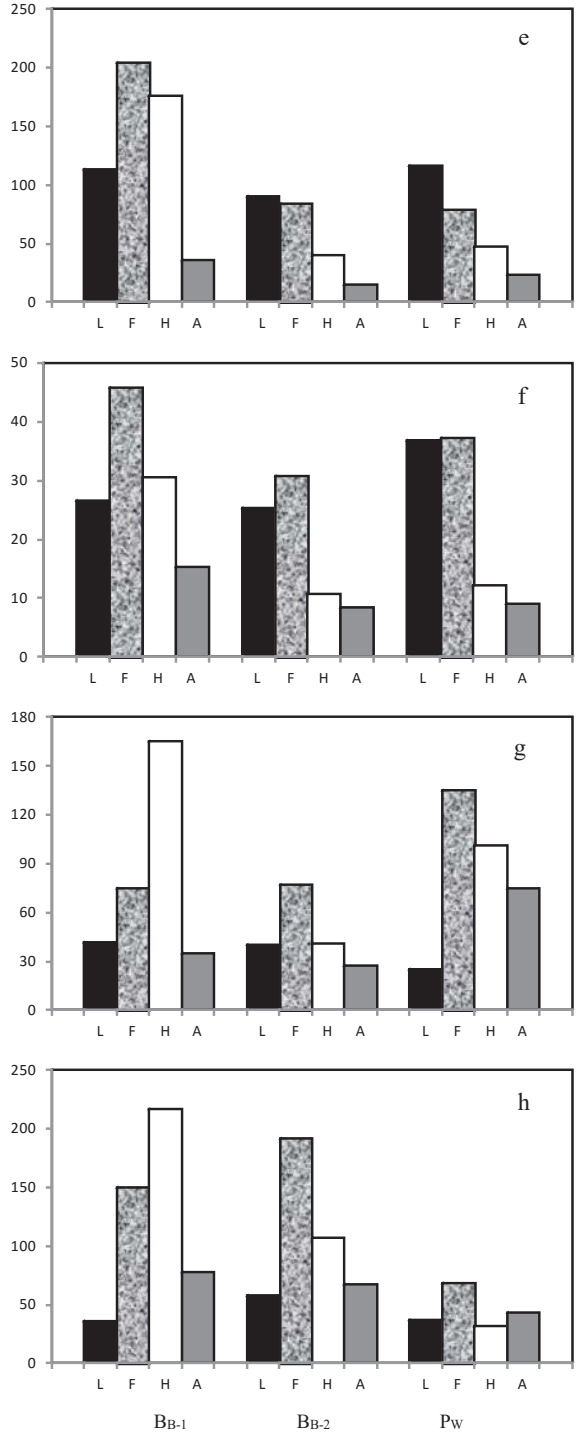


Fig. 4 (b) Soil enzyme activities in each horizon e: phosphomonoesterase, f: phosphodiesterase, g: chymotrypsin, h: trypsin

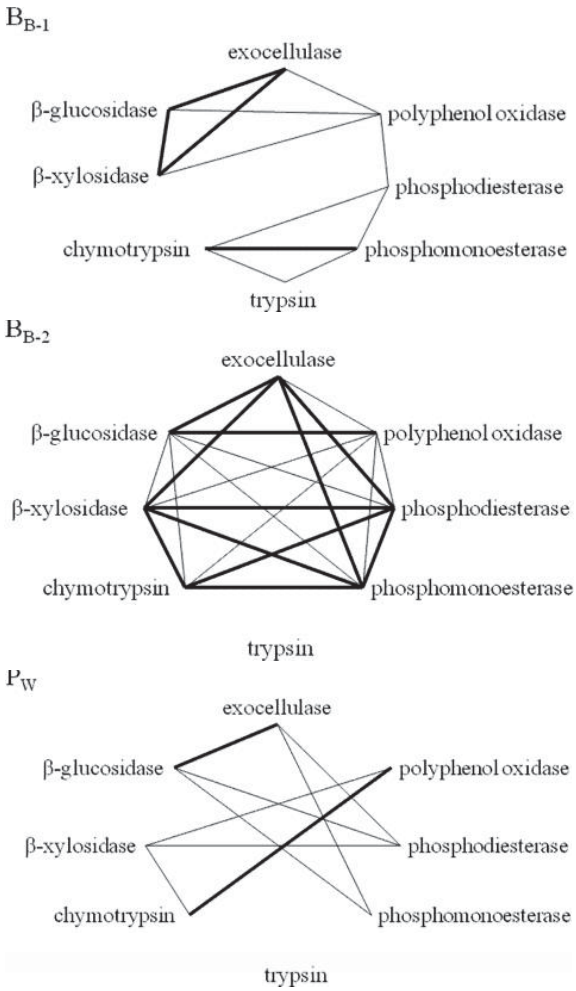


Fig. 5 Inferred network between enzymes in each soil type Edges between the soil enzymes indicate strong associations (>0.8) between soil enzymes. Bold lines indicate associations with a probability of 1% significance

## IV. Conclusions

In this study, we measured the activity of eight enzymes in each horizon of soil samples taken from Kasuga-yama Hill Primeval Forest and calculated Spearman's rank correlation to infer interactions among these enzymes. As these eight enzymes are commonly found in the forest soil, determining the relevance of their interactions will help inform our understanding the overall soil ecosystem.

Enzyme activities revealed that $B_{B-1}$ samples had a faster metabolism than $B_{B-2}$ samples, and that this was influenced by presence of forest vegetation. Given the relationships between the soil enzymes calculated by Spearman's rank correlation, activities of soil enzymes from $B_{B-2}$ samples were deemed more complex than those from $P_w$ samples.

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