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SOIL MICROBIOLOGY

High Abundance and Role of Antifungal Bacteria in Compost-Treated Soils in a Wildfire Area

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Abstract Compost has been widely used in order to promote vegetation growth in post-harvested and burned soils. The effects on soil microorganisms were scarcely known, so we performed the microbial analyses in a wildfire area of the Taebaek Mountains, Korea, during field surveys from May to September 2007. Using culture-dependent and -independent methods, we found that compost used in burned soils influenced a greater impact on soil fungi than bacteria. Compost-treated soils contained higher levels of antifungal strains in the genera *Bacillus* and *Burkholderia* than non-treated soils. When the antifungal activity of *Burkholderia* sp. strain O1a_RA002, which had been isolated from a compost-treated soil, was tested for the growth inhibition of bacteria and fungi isolated from burned soils, the membrane-filtered culture supernatant inhibited 19/37 fungal strains including soil fungi, *Eupenicillium* spp. and *Devriesia americana*; plant pathogens, *Polyschema larviformis* and *Massaria platani*; an animal pathogen, *Mortierella verticillata*; and an unidentified Ascomycota. However, this organism only inhibited 11/151 bacterial strains tested. These patterns were compatible with the culture-independent DGGE results, suggesting that the compost used in burned soils had a greater impact on soil fungi than bacteria through the promotion of the growth of antifungal bacteria. Our findings indicate that compost used in burned soils is effective in restoring soil conditions to a state closer to those of nearby unburned forest soils at the early stage of secondary succession.

Abbreviations

DGGE	Denaturing gradient gel electrophoresis
C/N	Ratio of carbon to nitrogen
OM	Organic matter
EC	Electrical conductivity
ApCFU	Approximate colony-forming unit
PCR	Polymerase chain reaction
OD	Optical density
TS	Tryptic soy
CS	Culture supernatant
FAME	Fatty acid methyl ester

Introduction

Fire is a prescribed or natural disturbance that resets the biological succession of a forest. Wildfire may be beneficial or detrimental over short- and long-term periods, depending on fire severity (intensity and frequency), soil conditions, and the soil's capacity to recover after disturbance [7, 37]. A large-scale forest fire often causes loss of life and damage to property, emission of large amounts of green-

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house gases into the atmosphere [7, 10, 47], and loss of soil function [1, 31, 37]. The short-term impacts will be greatest on the soil surface and in the upper mineral soil horizon because the fire incinerates plant materials, humus, detritus, and soil organisms, thereby resulting in alterations in soil properties and community structures.

A post-fire forest soil is likely to be inhabited by transient microorganisms that cause soil conditions to become suitable for mutual interactions between soil microbes and plant species via food webs [19]. The species diversity depends on weather conditions (e.g., water content and temperature), soil properties, and nutrients released from dead organisms and plant materials. These biological and environmental factors can influence the mortality and survival rates of soil microorganisms following a fire. There have been several studies concerning the disturbance of soil communities and their functions in prescribed fire or wildfire soils [11, 19, 25, 28, 45, 54] and in tall grass prairie [35]. Because soil microbial community structures are varied in time and space, it will take a long time, from several years to decades, to establish a stable community structure. Yet, little has been studied for biological control process of soil microorganisms in the post-fire degradation or regeneration processes.

We studied the effects of compost used in burned soils during a summer period from May 11th to September 29th 2007. During field surveys, vegetation was investigated, and soil microorganisms in the study plots and nearby unburned forest sites were analyzed by culture-dependent and -independent manners. Significantly, we found that the compost used in burned soils was effective in controlling soil fungi and restoring soil conditions to a state closer to those of nearby unburned forest soils. Lastly, we discussed that the abundance of antifungal strains, which are able to inhibit the growth of suspected soil pathogens such as *Polyschema larviformis*, *Massaria platani*, and *Mortierella verticillata*, could benefit for the early recovery of vegetation and fauna in burned soils.

Materials and Methods

Location of Experimental Patches and Soil Sampling

A large-scale forest fire took place on April 29, 2007 in a 20–30-year-old regenerated secondary forest area of the Taebaek Mountains, Uljin, Korea. The fire burned an estimated 2 km^2 of temperate deciduous forest around several mountains on the steep east sea-facing slopes for 34 h before being extinguished. The first survey was conducted on May 11, 2007. At that time, we set up four 12×12-m study plots on the southeast slope (36°51′41″ N, 129°23′56‴ E; elevation 330 m a.s.l.) of Hyunjong Mountain (413 m a.s.l.), where most trees, plant debris, and soils were degraded by intensive fire. Replicate O plots were set up with a distance of 6 m, and the surface (144 m^2) of each plot was treated with approximately 30 kg of compost. As controls, two non-treated C plots were set up horizontally 12 m apart from the O plots.

Preparation of Compost

Compost used in this study was prepared with the inoculation of pre-cultured *Saccharomyces* sp. (4.3×10^2 *c*olony-*f*orming *u*nits g^{-1} dry slurry), *Rhodopseudomonas* sp. (4.8×10^2 *cfu*), and *Lactobacillus* sp. (1.9×10^7 *cfu*) because they did not cause odor problems during composting (data not shown). These microorganisms (2.4 g dry weight) were inoculated into 10 kg of organic waste materials of sugar cane (30 g), rice bran (6 kg), oil meal (3 kg), fish meal (0.5 kg), and bone meal (0.5 kg). The mixture was homogenized by a ball mixer, poured into a container with a cover, and allowed to age for about 1 month at ambient temperature till the C/N reached about 27. The resulting product was analyzed by the Methods of Soil Analysis [46]: 30.6% (*w*/*w*) total organic carbon, 1.13% (w/*w*) total nitrogen, 37.2 g kg^{-1} total phosphate, 9.78 g kg^{-1} potassium (K^+), 1.21 g kg^{-1} sodium (Na^+), 1.36 g kg^{-1} calcium (Ca^{2+}), and 2.29 g kg^{-1} magnesium (Mg^{2+}). Its preliminary use on a grassland showed no apparent toxicity on the growth of grass sprouts (data not shown).

Vegetation Survey

The field survey for vegetation classification was conducted on September 29, 2007. Each species was identified and quantified using the Braun–Blanquet cover scales [6]. Heights of 12 shoot apices of *Lespedeza bicolor* were measured as an indicative level of vegetation growth in each plot because this shrub was predominant in the post-fire forests during the summer.

Soil Sampling and Analysis

Soil samples were collected in May and September 2007. In a 12×12-m plot, sampling was based on a spatially stratified, random sampling approach: each plot was divided into equal sectors of 1 m^2, and ten sectors were randomly selected to collect about 500 g of soil from the upper 2 cm of the profile. Plant debris, charcoals, and >2-mm gravels were carefully removed using sterile scoops and sieves. Soil subsamples obtained from each sector were thoroughly mixed in a sterile sampling bag. In the same mountain, two soil samples were taken as references from nearby unburned forest (NF) sites which had similar

conditions of altitude and slope. Homogenized soil samples were divided and stored at 4°C for sampling representative microorganisms within 24 h or at −80°C before use for soil DNA extraction or chemical analysis. Chemical analyses of soil properties such as pH of bulk soil, % moisture, % organic matter (OM), total nitrogen, and electrical conductivity were carried out independently for three times as described elsewhere [26], and the results were reported as means and standard deviations.

Sampling and Colony Counts of Cultivable Microorganisms

Five grams (wet weight) of homogenized soil was used for sampling representative soil microorganisms by the dispersion and differential centrifugation method as described elsewhere [23, 29]. Tenfold serial dilutions of soil extract were prepared with sterilized phosphate-buffered saline on ice, and aliquots (1 mL) corresponding to the dilution ratios of 10^{-3} to 10^{-5} g dry weight soil were evenly spread on the triplicate of R2A agar (BD Diagnostic System, Sparks, MD, USA) plates supplemented with 100 μg mL^{-1} cycloheximide or starch-casein (SC; [3]) agar plates with 100 μg mL^{-1} cycloheximide, or potato-dextrose (PD; BD Diagnostic System) agar plates with 100 μg mL^{-1} ampicillin+30 μg mL^{-1} chloramphenicol+10 μg mL^{-1} kanamycin for the enumeration of bacteria and fungi cultivable under the selective pressures of antibiotics. Plates sealed with parafilm were incubated at 20°C for 21 days, and colony numbers (CFU (g dry weight soil)$^{-1}$) were counted daily for the calculation of approximate colony-forming unit (ApCFU) using a SigmaPlot program version 10.0 (Systat Software, Inc., Chicago, IL, USA) with an iterative curve fitting equation: $CFU_{obs} = ApCFU/(1 + \exp(-k(t - t_{1/2})))$. The CFU_{obs} is an observed colony number at time t, k is a specific constant (d^{-1}) for colony formation, and $t_{½}$ (d) is a time constant for half-maximum colony count, i.e., ½ApCFU at time $t_{½}$.

Strain Isolation and DNA Analysis

About 20–30 colonies were isolated from every colony-counting plate and were successively (two to three times) transferred to fresh media without addition of antibiotics. Pure strains were observed by morphology under a microscope. To infer the taxonomic groups of strains, genomic DNA was extracted using a Wizard genomic DNA extraction kit (Promega, Madison, WI, USA) and was used for the amplification of bacterial 16S rRNA and fungal 28S rRNA gene fragments by polymerase chain reaction (PCR) with the universal primers: BAC27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) [22] and BAC1390R (5′-GAC GGG CGG TGT GTA CAA-3′) [55] for eubacteria; ACT253F (5′-GCG GCC TAT CAG CTT GTT-3′) [51] and BAC1390R for actinobacteria; and LR0R (5′-ACC CGC TGA ACT TAA GC-3′) [49] and LR5 (5′-TCC TGA GGG AAA CTT CG-3′) [49] for fungi. The PCR was performed with a Dyad DNA Engine thermo cycler (MJ Research, Inc., Waltham, MA, USA) as follows: 250 ng of genomic DNA, 50 pmol each of appropriate primers, 200 μmol each of deoxyribonucleoside triphosphates, 1 μL of acetylated bovine serum albumin (20 mg/mL, Promega, Madison, WI, USA), 5 U of ExTaq polymerase (Takara Korea, Seoul, Korea), and 5 μL of l0×ExTaq buffer in a 50-uL reaction mixture. The PCR tubes were heated to 94°C for 2 min and were then followed by 25 cycles, carried out at 92°C for 30 s, 52°C for 1 min, and 72°C for 2 min. According to the morphology and PCR results, the strains were divided into five groups: (a) filamentous actinobacteria which form mycelium, (b) non-filamentous actinobacteria which do not form mycelium, (c) other eubacteria, (d) fungi, and (e) mixed fungi and bacteria. Except for mixed fungi and bacteria, PCR-amplified 16S/28S rDNA products were cut into fragments using restriction enzymes, *Hae*III and *Rsa*I, in order to subdivide strains. After agarose gel separation, ethidium bromide (EtBr)-stained band positions, which were corrected for every gel with three lanes of 25 bp/100 bp DNA markers (Bioneer, Seoul, Korea), were compared between lanes by an image analyzer program (UVP Inc., Upland, CA, USA). The diversity was calculated by the Shannon index, $H=\sum -p_i \ln(p_i)$, in which p_i is the normalized and the average ratio of members of i subgroup to the total strains obtained from the pooling of replicate plots and selective media with the same regime of compost treatment. Evenness scale (J) was calculated by the equation, $J=H/H_{max}$, in which H_{max} is the maximum diversity expressed by logarithm of the total number of subgroups (S), i.e., $H_{max}=\ln(S)$. Partial 16S/28S rDNA sequences of selected antifungal strains were determined by an Applied Biosystems 3730 DNA analyzer.

Denaturing Gradient Gel Electrophoresis

To analyze soil microbial populations by a culture-independent method, denaturing gradient gel electrophoresis (DGGE) separation of PCR-amplified 16S and 28S rDNA products was performed using soil DNA extracts as described elsewhere [44]. Two sets of 0.25-dry-weight-gram samples of soil from each plot were subjected to DNA extraction using a PowerSoil® DNA isolation kit (MO BIO Lab., Inc., Carlsbad, CA, USA). Extracted DNA concentration was determined by measuring the absorbance at 260 nm (extinction coefficient of DNA, ε_{260}=0.02 μg^{-1} cm^{-1}) when the $A260/A280$ value was about 1.8. For each set of soil DNA extracts, equal amounts of DNA obtained from replicate plots were combined and used as templates for PCR

amplification of bacterial 16S rDNA with the primers (GC)-341F (5′-(GC) CCT ACG GGA GGC AGC AG-3′) [32] and 534R (5′-ATT ACC GCG GCT GCT GG-3′) [32] and for PCR amplification of fungal 28S rDNA with the primers U1 (5′-GTG AAA TTG TTG AAA GGG AA-3′) [41] and (GC)-U2 (5′-(GC) GAC TCC TTGG TCCGTGTT-3′) [41]. For DGGE, a 40-bp (GC)-clamp, 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′ [32], was attached to the 5′-ends of 341F and U2. Using the appropriate set of primers mixed in the same composition as described above, the PCR reactions were carried out with a hot start at 94°C for 2 min, followed by 35 cycles of 92°C for 15 s, 52°C for 1 min, and 72°C for 1 min. PCR products were separated in 9% (wt/vol) polyacrylamide gels with a linear gradient of 30% to 60% denaturant (100% UF, 7 M urea, and 40% formamide) in 0.5×TAE according to the method of Hayes et al. [20]. After electrophoresis at 59°C and 200 V, the gels were stained with EtBr, washed with water, and photographed by a Gel Logic200 Imaging System (Carestream Health, Inc., Rochester, NY, USA).

Antimicrobial Activity Screening

Strains, which had the ability to inhibit the growth of fungi, were tested using agar culture disks (5 mm in diameter) on 0.2-μm nitrocellulose membranes (47 mm in diameter) laid on 0.8% agar lawns of *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC29213, and *Candida albicans* IMSNU30018. These three strains were selected from various standard strains of Gram-negative bacteria, Gram-positive bacteria, and fungi because they gave reliable results from the test conditions (data not shown). Agar lawns were prepared with the cell suspensions at an optical density (OD_{600}) of 0.1, which were mixed with equal volumes of double-strength tryptic soy (TS) medium (BD Diagnostic System) containing 1.6% molten agar by stirring at about 40°C. About 2 mL of 0.8% molten agar was poured on standard TS agar plates (diameter, 85 mm; 20 mL volume of media) and solidified for 2 h under a sterile bench. Then, a sterile 0.2-μm membrane was placed on the agar plate. Agar disks of strains cultivated for 1 or 4 weeks were cut out aseptically and placed on eight radial positions of the membrane. A control agar disk without cells was placed on the middle of the membrane. After 24 h of incubation at 37°C, the agar disks and membrane were removed in order to measure the diameter of clear zones beneath the membrane.

Antifungal Strain Tests

Strain O1a_RA002, a member of the most abundant *Burkholderia* strains able to inhibit the growth of *C. albicans* in a collection of cultivable microorganisms from compost-treated soils, was selected to examine the production of effects for the inhibition of growth of bacteria and fungi. During cultivation with TS broth media at 20°C and 150 rpm, the culture supernatant (CS) of strain O1a_RA002 was taken daily and filter-sterilized through 0.2-μm membrane filtration. To test against *C. albicans*, 50 μL of CS was mixed with an equal volume of exponentially growing *C. albicans* cells in TS media at OD_{600} of 0.1 in microplate wells and then incubated for 6 h at 37°C and 100 rpm. The OD_{600} values of CS-treated (OD_{CS}) and non-treated (OD_{TS}) cultures were measured using an ELx808™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The percent growth inhibition of *C. albicans* was determined by the following equation: $\%\,\text{growth inhibition} = \{(OD_{TS} - OD_{CS})/OD_{TS}\} \times 100$. A near-maximum activity of strain O1a_RA002 for growth inhibition of *C. albicans* was obtained at day 5 during cultivation, and the CS was tested against pure cultures of 151 bacteria and 37 fungi isolated from burned soils in May 2007. Isolated strains were exponentially grown with TS broth media at 20°C and 100 rpm. For testing, the cell densities (OD_{600}) were adjusted to 0.1 with 50 μL of TS media in microplate wells, to which an equal volume of CS or TS was then added. At the initial time and 24 h after the cultivation of microplates at 20°C and 100 rpm, the OD_{600} values of CS-treated (OD_{CS}) and non-treated (OD_{TS}) cultures were determined with a microplate reader. More than twofold difference in the ratio of ΔOD_{CS} $(OD_{CS(24\,h)} - OD_{CS(0\,h)})$ to $\Delta OD_{TS}(OD_{TS(24\,h)} - OD_{TS(0\,h)})$ was considered to be significant. A "positive effect" was defined with more than 200% population growth, $(\Delta OD_{CS}/\Delta OD_{TS} > 2)$; a "null effect" was considered if $\Delta OD_{CS}/\Delta OD_{TS}$ ranged between 0.5 and 2; and a "negative effect" was recognized if there was more than 50% growth inhibition $(\Delta OD_{CS}/\Delta OD_{TS} < 0.5)$.

Fatty Acid Profiles and Numerical Tests for Bacterial Strains

To obtain the metabolic and biochemical characteristics of selected bacteria, numerical tests were performed using API 20E, API 20NE, API ZYM , and API Coryne test kits (bioMerieux, Inc., Hazelwood, MO, USA) for non-actinobacteria and actinobacteria, and fatty acid methyl ester (FAME) composition was analyzed using a Hewlett-Packard 6890 gas chromatograph installed with Microbial Identification Software (MIDI, Newark, DE, USA).

Nucleotide Accession Numbers

The sequences reported in this study have been submitted to the GenBank database under accession numbers FJ800554 to FJ800564 and GU188571 to GU188586.

Statistical Analysis

Statistically significant differences between treatments were tested by analysis of variance (ANOVA) and *t*-test with the significance $p<0.05$.

Results

Vegetation Recovery in Post-fire Forest Soils

Several species of forbs and trees grew in the post-fire forest soils during summer (Table 1). At a glance, compost-treated O plots showed higher recoveries of vegetation (five tree species and eight to ten forb species) than non-treated C plots (three to four tree species and six to nine forb species). The O plots resulted in higher abundance and cover scales of most plant species, except for *Aster scaber* which was found only in the C plots. At the stage of shrubland, the O plots each gave higher shoot heights of *L. bicolor* ($n=12$ from each plot) than the C plots (ANOVA *t*-test, $p<0.05$). *L. bicolor* is an indigenous species which is occasionally dominantly grown in barren pine forest soils during summer in Korea. Some buried seeds of this tree could survive and grow out from the underground soils at the stage of forest regeneration post-fire.

Soil Properties

The soil texture in the study area was a sandy silt loam. The four experimental plots showed no significant difference in the pH of bulk soils during summer period (Table 2). After fire, the soil pH tended to increase overall probably due to the ash deposition.

Compared to non-treated soils, compost-treated soils resulted in a significant increase of organic matter (%OM) after summer (ANOVA *t*-test, $p<0.05$). The compost-treated soils also had slightly higher water content than the non-treated soils, even though the difference was not statistically significant ($p>0.05$), probably because of the frequent rainfalls during the samplings in September 2007. The application of ~30 kg compost into 144 m^2 surface of soil was accounted for ~0.1% OM increase at maximum from the sampling depth of 2 cm, so it was calculated that the larger increase of OM in compost-treated soils was mainly due to vegetation growth and release of organic carbon compounds from plants into the soils. A net increase of OM appeared to be positively related to the extent of enhanced electrical conductivity ($\mu S\ cm^{-1}$) in the order of non-treated, compost-treated, and NF soils.

The compost application did not result in a significant increase of total nitrogen in burned soils after summer. Both compost-treated and non-treated soils contained much lower N levels than NF soils. In this work, we noticed that

Table 1 Braun–Blanquet cover index of plant species and average heights of *L. bicolor* in Korea's burned forest sites

Plant	Species	Experimental plots			
		C1	C2	O1	O2
Tree	*Lespedeza bicolor*	3	2	3	3
	Quercus dentate	1	1	2	2
	Quercus serrata	1		1	2
	Pinus densiflora	1	1	1	2
	Zanthoxylum schinifolium			1	1
Forb	*Artemisia japonica*	1	1	1	1
	Aster scaber	1	1		
	Carex humilis		1	2	2
	Commelina communis	r		1	1
	Miscanthus sinensis	1	1	3	3
	Patrinia villosa	1		1	1
	Pueraria thunbergiana	r		1	
	Sanguisorba officinalis	r	1		1
	Setaria faberi			1	2
	Smilax china	1		2	
	Spodiopogon cotulifer	1	2	2	2
	Themeda triandra v. japonica			1	
Heights of *L. bicolor* (mean±SD), cm		100±13	63±11	119±13	129±14

Values are scales from replicate study plots: O, compost-treated plots; C, non-treated plots. Cover index [8]: blank, not detected; *r*=rare individuals; 1=1–5% cover; 2=6–25% cover; 3=25–50% cover

Table 2 Properties of burned forest soils and nearby unburned forest soils in Hyunjong mountain, Uljin, Korea

Sampling dates	Plots	Bulk pH	Water content (%)	Organic matter (%)	Electrical conductivity (μS/cm)	Total Kjeldahl nitrogen (mg/kg d.w. soil)
May 2007	C (n=6)	4.9±0.3	12.8±0.6c	5.0±0.4c	34±11.4c	1,207±295c
	O (n=6)	5.3±0.2ac	12.8±0.6c	6.6±1.2c	47±6.3c	1,053±50b
September 2007	C (n=6)	5.3±0.1c	20.3±1.6ac	5.5±0.7c	41.9±7.5c	808±182c
	O (n=6)	5.3±0.1c	22.3±0.9a	8.4±0.4ab	79.4±2.9abc	1,064±71bc
	NF (n=6)	4.6±0.2b	26.9±3.9ab	10.2±1.3ab	142±6.8ab	1,990±289ab

Values are averages±SD from the means of triplicate measurements from replicate study plots: O, compost-treated plots; C, non-treated plots; NF, unburned forest soils. Significant values (p<0.05) are shown with letters: a, comparison with C plots in May 2007; b, comparison with C plots in September 2007; c, comparison with NF soils

compost-treated soils had higher N levels than non-treated soils (p<0.05). The difference of about 250 mg N (d.w. kg soil)$^{-1}$ between them was greater than the N supply from compost that was calculated to be about 50 mg N (d.w. kg soil)$^{-1}$. It is likely that the compost-treated soils increased the capacity to maintain soil N levels possibly with enhancing the microbial activity for nitrogen fixation and assimilation, e.g., through rhizobial bacteria and nitrifying bacteria, which are likely to associate with plant growth.

Population Analysis of Soil Microorganisms

Sampling soil microorganisms from the four study plots and NF sites gave similar ranges of 1.8×10^6 to 1.2×10^7 cfu (g dry weight soil)$^{-1}$ irrespective of the compost treatments ("Electronic Supplementary Material", Table S1). Interestingly, it was found that, among cultivable microorganisms, the percent levels of filamentous fungi in compost-treated soils were much lower than those in non-treated soils (Fig. 1).

A total of 433 bacterial strains and 128 fungal strains collected from soils were subdivided into 177 subgroups, including 134 bacterial subgroups and 43 fungal subgroups, based on the restriction fragment patterns of 16S/28S rDNA (Fig. 2). However, the number of subgroups obtained from each plot was too small to calculate the diversity (H) index, and the composition of microorganisms was too varied between plots because there seemed to be heterogeneities even in small soil patches. To solve this under-sampling problem and patchiness, strains obtained from replicate plots using different media for selection of bacteria and fungi were pooled into the same category to calculate the normalized, average ratios (p_i) of subgroups. By this method, we found that the diversity (H) scales of soil microorganisms in burned soils were decreased during summer, largely because the unique (heterogeneous) microorganisms were decreased from 70.6% to 56.5% (Table 3). The evenness (J) scales were also lowered after summer in burned soils since members of a few subgroups (B3, B9, and B17) were accounted for >30% of the total isolates (see Fig. 2 left panels).

In Fig. 3, the DGGE fingerprints of bacterial 16S rDNA and fungal 28S rDNA showed that fungal populations were markedly changed in compost-treated soils, being similar to those of NF soils. In contrast, it appeared that soil bacteria were perhaps affected by weather (temperature, precipitation, etc.) rather than compost and soil sources and fluctuated in the composition and number of populations to lesser extents than fungi. The compositions of dominant bacteria were different between burned soils, C_b and O_b, maybe due to variations in the degree of degradation by fire. After summer, however, the bacterial compositions in compost-treated (O_a) and non-treated soils (C_a) were similar regardless of compost application. Accordingly, the compost used in burned soils had a greater effect in controlling soil fungi than bacteria. The results from culture-dependent and -independent experiments overall showed that compost-treated soils were more potent to restore soil microorganisms, at least in part, to a state close to those of NF soils than non-treated soils.

Antifungal Activity-Producing Strains

Membrane-agar diffusion assays were used to screen and test antifungal strains using agar culture disks against *E. coli*, *S. aureus*, and *C. albicans*. Among 561 strains tested, the members of 11 subgroups, including two unidentified fungi, were able to produce antifungal activities into the media consistently at 1 and 4 weeks during cultivation (Fig. 4a). By sequencing of PCR-amplified partial 16S and 28S rDNA products, the selected strains were tentatively classified to members of the genera *Burkholderia* (subgroups B17, B85, and B86), *Bacillus* (B22), *Streptomyces* (B3 and B129), and *Eupenicillium* (F4 and two not determined subgroups), and their abundances in each plot were calculated to obtain the percent ranges in Table 4. Amongst them, the members of subgroups B3 and B17

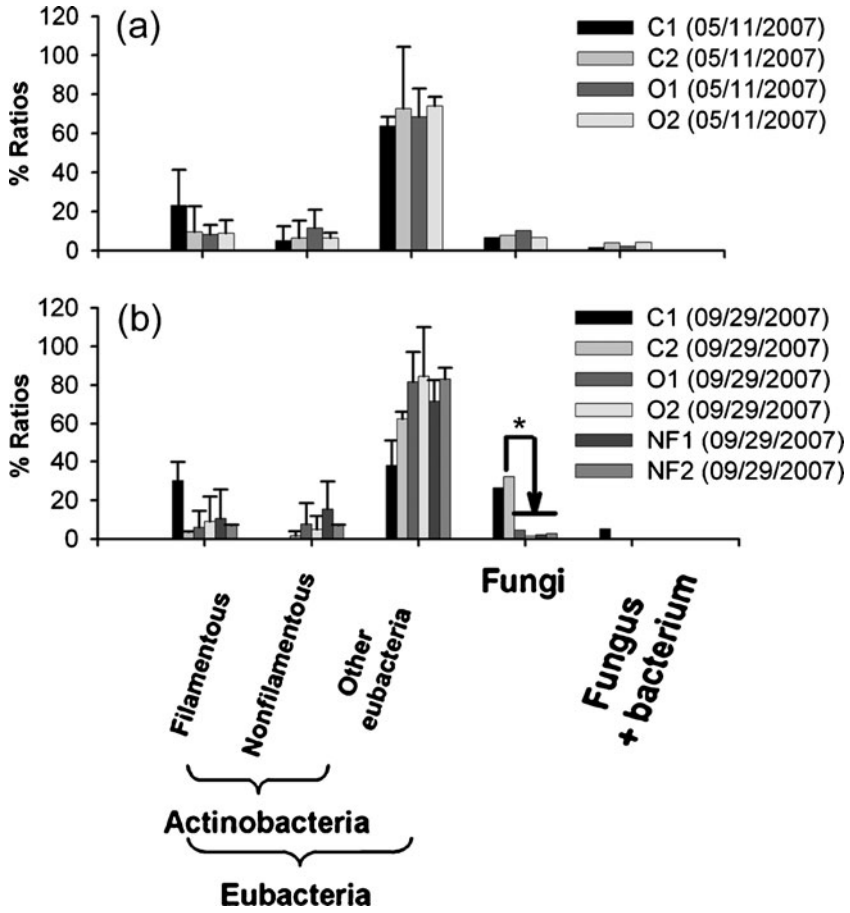

Figure 1 Percentages of taxonomic groups of isolated microorganisms. **a** Soil samples collected from burned forest sites in May 2007. **b** Soil samples collected from burned and nearby unburned forest (*NF*) sites in September 2007. A statistically significant difference (*t*-test, $p<0.05$) is shown with an *asterisked arrow*

were most abundant in non-treated and compost-treated soils, respectively, as seen in Fig. 2.

Members of *Burkholderia* B17 were able to produce a specific activity for the inhibition of growth of *C. albicans*, whereas the specific activities of *Streptomyces* B3 members were varied depending on strains, which could inhibit both, either, or none of *E. coli* and *S. aureus*. The specific activity of fungi *Eupenicillium* strains was similar as that of *Burkholderia* B17 members. However, the fungal levels in compost-treated soils were much lower than the bacterial levels. Soil *Bacillus* strains, which were efficacious to inhibit fungi and bacteria broadly, were also increased after compost treatment in burned soils. Based on the abundance and distribution of antifungal strains, it was expected that members of the genera *Burkholderia* and *Bacillus* might play a role in controlling soil microorganisms.

Antifungal Activity of a *Burkholderia* Strain

It was needed to investigate the effects of antifungal strains on the growth of fungi and bacteria in burned soils. To perform this experiment, strain O1a_RA002, which belongs to *Burkholderia* subgroup B17, was selected and tested retrospectively for 37 fungal strains and 159 bacterial strains collected from burned soils in May 2007. Strain O1a_RA002 was able to produce an antifungal activity constitutively during cultivation with tryptic soy media. A near-maximum activity was obtained at day 5, which was accounted for about 80% growth inhibition of *C. albicans* (Fig. 4b).

The added CS resulted in more than twofold growth inhibition of 19 out of 37 fungal strains tested. The severely inhibited fungi, *Eupenicillium* spp. (subgroups F2, F3, F4, and F8), plant pathogens *P. larviformis* (F5) and *M. platani* (F6), an animal pathogen *M. verticillata* (F7), a thermotolerant soil hyphomycete *Devriesia americana* (unidentified subgroup), and an Ascomycota species (F14) are mostly known as hypogeous (underground) fungi which are likely to dwell in soils (Table 5). In contrast, the less affected fungi belong to members of hypergeous (aerial) fungi; *Allantophoma endogenospora* (F1), *Melanopsammella* sp. (F9), and *Aleurodiscus farlowii* (unidentified subgroup). On the other hand, the added CS only inhibited 11/159 bacterial strains. The consequence of this inhibition was the decrease of fungi-to-bacteria ratio as observed in compost-treated soils. Virtually, the severely inhibited fungi were not found within a range of 10^{-3} to 10^{-5} dry weight gram of compost-treated soils, where strains of *Burkholderia*

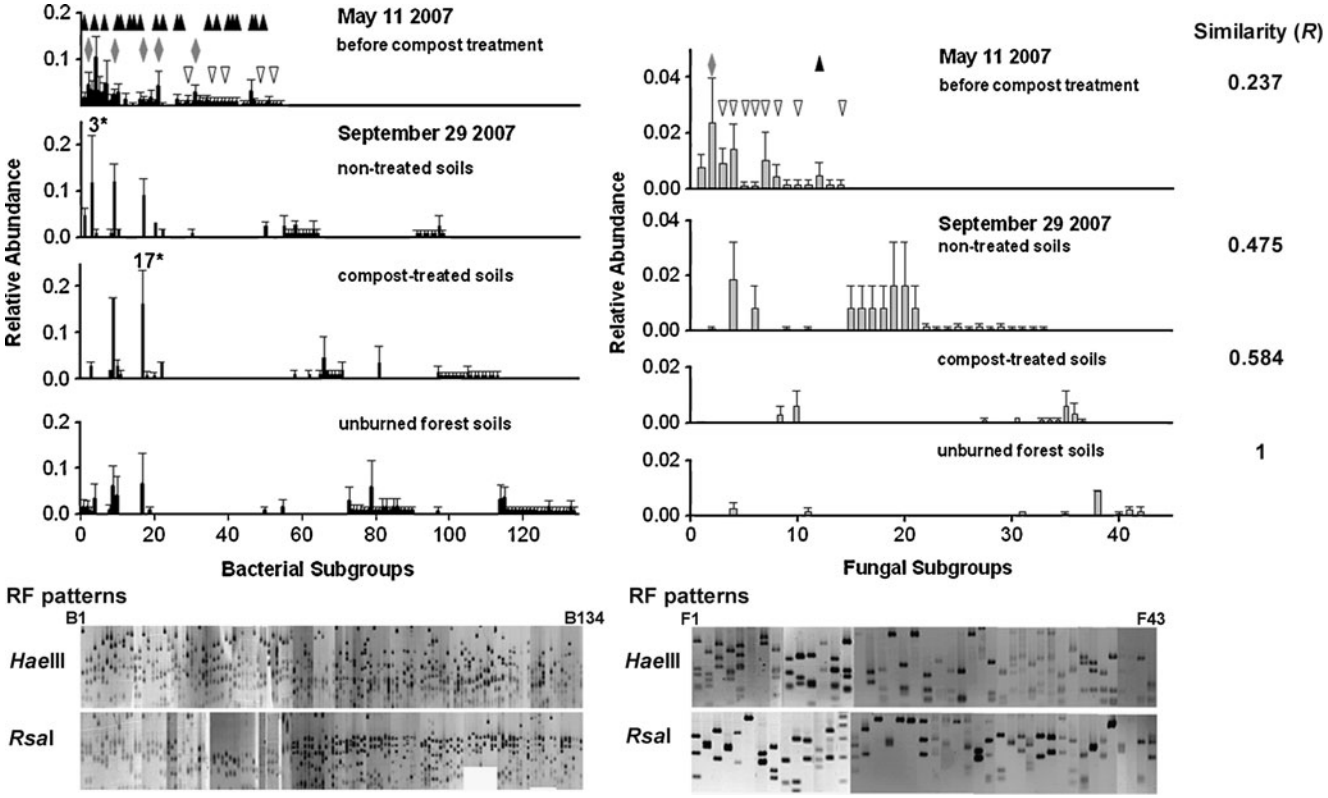

Figure 2 Relative abundance of cultivable bacteria (*left panels*) and fungi (right panels) obtained from burned and nearby unburned forest soil samples. The average ratio and standard deviation (*error bar*) of each subgroup was calculated from the pooling of strains obtained from replicate plots and selective media with the same regime of compost treatment. *Hae*III and *Rsa*I restriction enzyme fragment patterns of each subgroup are shown in the *lower part* of the figures. Negative (*unfilled inverted triangles*) or positive (*filled triangles*) effects of antifungal strains, O1a_RA002, on the growth of soil microorganisms were tested using bacteria and fungi isolated from burned soils in May 2007, and ambiguous results are shown with *gray diamond symbols*. The largest bacterial subgroups, B3 and B17, are *asterisked* over compost-treated and non-treated soils, respectively. Pearson correlation analyses of the abundances of both bacterial and fungal subgroups with those of unburned forest soils result in similarity (*R*) values at the *left column*

B17 were abundant (see Fig. 2). This finding was compatible with the DGGE results that the compost used in burned soils caused a greater impact on soil fungi than did on bacteria.

Numerical and Fatty Acid Analyses of Antifungal Bacteria

Numerical tests using API 20E, 20NE, and ZYM tests were performed in order to describe the metabolic properties of the antifungal strains. Three *Burkholderia* strains all showed positive results from API 20E and 20NE tests for acid/alkaline phosphatases, β-galactosidase, acetoin production, myristate (C14) lipase, and lysine decarboxylase but differed from each other in β-glucosidase, nitrate reduction, ornithine decarboxylase, and adipate assimilation. From the same tests, two *Bacillus* strains were both positive for arginine dihydrolase but differed in nitrate reduction. Three *Streptomyces* spp. strains were distinguished by nitrate reduction and glucose fermentation using API Coryne tests. The numerical results are summarized in the "Electronic Supplementary Material", Table S2.

Table 3 Total subgroups and unique subgroups of cultivable microorganisms isolated from burned and nearby unburned forest (NF) soils

Sampling dates	Soil samples (number of plots)	Number of strains	Total subgroups	Unique subgroups	Shannon index (*H*)	Evenness index (*J*)
May 2007	Before treatment (4)	195	68	48 (70.6%)	3.0259	0.7171
September 2007	Non-treated soils (2)	119	53	30 (56.6%)	2.2273	0.5610
	Compost-treated soils (2)	120	46	26 (56.5%)	2.4593	0.6424
	NF soils (2)	120	59	42 (85.6%)	3.0074	0.7376

Percent levels of unique subgroups are in parentheses

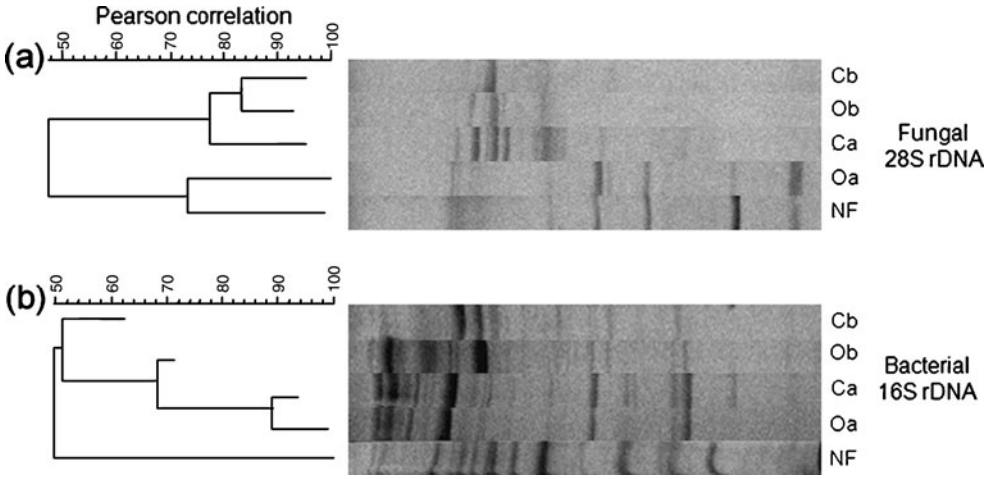

Figure 3 DGGE separation and cluster analyses of PCR-amplified products of **a** fungal 28S rDNA and **b** bacterial 16S rDNA obtained from the pooled samples of burned soils (C_b and O_b) in May 2007 and compost-treated and non-treated soils (O_a and C_a, respectively) in September 2007. As position markers, a combined sample of two nearby unburned forest (*NF*) soils was included in the replicate analyses. Similarity (%) between lanes was calculated as Pearson correlation coefficient of densitometric values by pixels with 5% position tolerance value. Cluster analysis was performed by neighbor joining method

Bacterial fatty acid methyl esters were analyzed in order to obtain the biochemical properties of the antifungal bacteria ("Electronic Supplementary Material", Table S3). The three genera were distinguished by the average percentages of the major FAME components: 17% $C_{16:0}$, 24% cyclo-$C_{17:0}$, 13% $C_{18:1}$ ω7c, and 19% $C_{19:0}$ cyclo ω8c for three *Burkholderia* strains; 8% iso-$C_{13:0}$, 7% anteiso-$C_{15:0}$, 8% 2OH-iso-$C_{15:0}$/$C_{16:1}$ ω7c, 31% iso-$C_{15:0}$, 11% iso-$C_{17:0}$, and 9% iso-$C_{17:1}$ ω10c and ω5c for two *Bacillus* strains; and 13% iso-$C_{14:0}$, 19% anteiso-$C_{15:0}$, 22% iso-$C_{16:1}$, 18% $C_{16:0}$, and 5% anteiso-$C_{17:0}$ for three *Streptomyces* strains.

Discussion

Manure and compost have been widely used to enhance soil fertility for vegetation in post-harvested or burned soils. Organic materials are of great interests since they function to protect soil erosion [18] and increase plant production more effectively than inorganic fertilizers [50]. Compost has been since long used to increase crop production and prevent soil loss through precipitation in agriculture. In burned soils, vegetation cover varies with soil organic matter, which is largely influenced by environmental

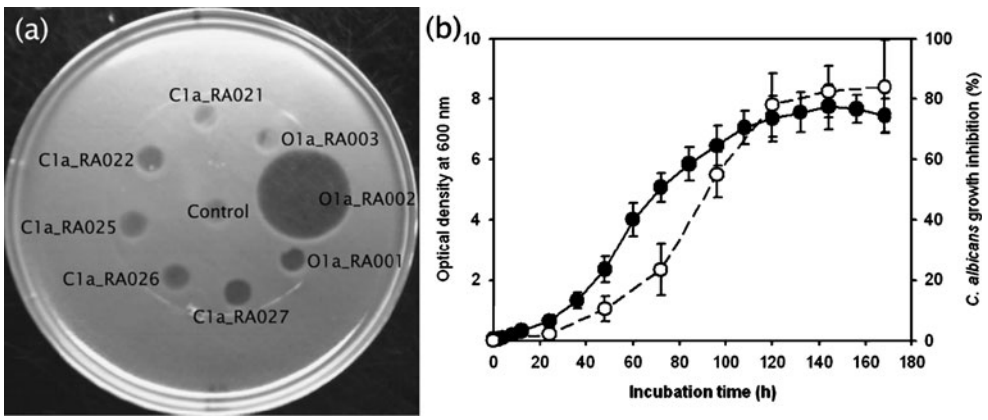

Figure 4 **a** A typical membrane–agar diffusion plate assay using 5-mm-diameter agar disks of R2A-cultivated strains. After 24 h of incubation, an agar disk of *Burkholderia* sp. strain O1a_RA002 shows an inhibitory effect on the growth of *C. albicans* by the formation of a large clear zone area compared to those of a control agar disk and others isolated from a compost-treated soil (*O1a_RAxxx*) and an untreated soil (*C1a_RAxxx*). **b** Growth curve of strain O1a_RA002 (*solid line*) and percent growth inhibition of *C. albicans* in TS broth media (*broken line*). The methods are described in the "Materials and Methods"

Table 4 Percent levels of antifungal strains among cultivable microorganisms isolated from burned soils and nearby unburned forest soils

Strain	Subgroup	GenBank accession number	Antimicrobial activity	Genus	May 2007		September 2007	
					C+O plots (4)	C plots (2)	O plots (2)	NF sites (2)
O1b_RA027	B3	FJ800562	SA, CA	*Streptomyces* (bacteria)	3.4±2.2	11.7±10.2	2.8±0.9	0.7±0.7
C1a_RA021	B3	FJ800554	EC, SA, CA					
NF2_SC017	B129	FJ800559	CA		ND	ND	ND	0.7±0.7
O1a_RA002	B17	FJ800561	CA	*Burkholderia* (bacteria)	1.3±0.8	8.9±3.7a	16.3±7.2ab	6.6±6.6
NF2_RA021	B86	FJ800557	CA		ND	ND	ND	1.7±1.7
NF2_RA022	B85	FJ800558	SA, CA		ND	ND	ND	1.7±1.7
O2a_RA019	B22	FJ800563	EC, SA, CA	*Bacillus* (bacteria)	0.4±0.4	1.7±0.1a	3.7±0.1ab	ND
O2a_RA020	B22	FJ800564	EC, SA, CA					
NF2_PD013	F4	FJ800556	SA, CA	*Eupenicillium* (fungi)	0.9±0.5	1.8±1.4	0.6±0.6	0.2±0.2
O1a_PD022	n.d.	FJ800560	CA					
NF1_PD011	n.d.	FJ800555	CA					

Percent levels are means±SD from the numbers of replicate plots in parentheses. Significant values ($p<0.05$) are shown with letters: a, comparison with burned plots in May 2007; b, comparison with non-treated C plots in September 2007. Subgroups are determined by the PCR restriction fragment analyses of 16S/28S rDNA. Results of antimicrobial activity assays are abbreviated

ND not determined, *SA Staphylococcus aureus*, *EC Escherichia coli*, *CA Candida albicans*

factors such as frequency and intensity of precipitation [13]. In disturbed soils with varying the regeneration type, the nitrogen availability is considered to be the most strongly associated characteristic for plant biomass production [38]. Thus, soil organic matter, water content, and nitrogen source may be critical factors which restrict the growth of plants and soil organisms in burned soils. In our study, compost used in burned soils helped to increase soil organic matter and maintain soil N levels along with vegetation growth in the first rainfall season after the fire. The life of

Table 5 Effects of culture supernatant of *Burkholderia* sp. strain O1a_RA002 on growth of soil fungi

Subgroups	Number of test strains	Nucleotide sequences of strains deposited at GenBank (accession no.)	BLAST searches (% similarity)	Effects of O1a_RA002	Habitat	Reference
F1	3	C1b_PD001 (GU188571); C1b_PD003 (GU188572); C2b_PD013 (GU188577)	*Allantophoma* spp. (95–96%)	Null for all three	Stem base/ litter	[14]
F2, F3, F4, F8	24	C2b_PD004 (GU188576); O1b_PD002 (GU188579); O1b_PD003 (GU188580); O1b_PD009 (GU188581); O1b_PD012 (GU188582); O2b_PD025 (GU188585)	*Eupenicillium* spp. (95–97%)	Negative for 13, null for 11	Soil/manure	[17]
F5	1	C1b_PD011 (GU188573)	*Polyschema larviformis* (96%)	Negative	Soil	[43]
F6	1	C1b_PD013 (GU188574)	*Massaria platani* (95%)	Negative	Soil	[42]
F7	2	C2b_PD001 (GU188575)	*Mortierella verticillata* (99%)	Negative for both	Soil	[24]
F9	3	O2b_PD002 (GU188583)	*Melanopsammella* sp. (97%)	Null for all three	Tree bark	[15]
F14	1	O2b_PD026 (GU188586)	Uncultured Ascomycota (97%)	Negative	Soil	
Unidentified subgroups	1	C2b_PD014 (GU188578)	*Devriesia americana* (97%)	Negative	Soil	[12]
	1	O2b_PD011 (GU188584)	*Aleurodiscus farlowii* (98%)	Null	Tree bark	[53]

Effects of strain O1a_RA002 are defined as "negative" for more than 50% growth inhibition of fungus; "null" for between 50% and 200% growth compared to the control set without addition of the filter-sterilized culture supernatant

soil microorganisms would be largely affected not only by heat stress from direct heat exposure but also by temporal changes in the soil conditions. In post-fire forest soils, the diversity and evenness of heterotrophic microorganisms could decrease with the loss of large portions of heterogeneous microorganisms together with the reduction of environmental heterogeneity and carbon and nitrogen sources. By contrast, some microorganisms could flourish with their capability of occupying most habitats and utilizing limited carbon and nitrogen sources in the disturbed soils.

Molecular ecological studies have been conducted to characterize soil microorganisms, which depend on the patterns of ribosomal RNA gene fragments generated by a culture-independent PCR. The DGGE method is among the simplest and most widely accepted platforms for the analysis of microbial communities in various environments [30, 34]. However, it is actually hard to distinguish the functioning elements from the DNA sequences or pattern analyses. The PCR primers used for amplification of bacterial rDNA and fungal rDNA only are limited to species with the conserved nucleotide sequences [32, 41]. If diverse DNA fingerprints are required, the number of PCR variants can be increased with the incorporation of inosine residues (or degeneracy) at ambiguous nucleotide positions of the PCR primers [52] or by the use of other primers targeted for less conserved internal transcribed spacer region primers [2, 6, 16]. A single-cell isolation and culture technique is yet the inevitable need in order to identify and characterize the truly functioning elements, albeit it has a limited view of only cultivable microorganisms. Our study shows that it is necessary to combine culture-independent and -dependent approaches such as DGGE fingerprints and conventional isolation and cultivation of microorganisms for understanding the abundance and role of antifungal strains in the environment. Together with the DGGE analysis, which overviews the microbial community structure, a single-cell isolation and cultivation is necessary for identification and characterization of a functioning microorganism which is able to control soil fungi effectively. Using routine isolation and cultivation techniques for heterotrophic microorganisms, we successfully found an antifungal *Burkholderia* strain O1a_RA002 from a compost-treated soil which was potent to control soil fungi, including suspected plant pathogens belonging to *P. larviformis* and *M. platani*.

Previously, it was reported that some strains of the genus *Burkholderia* were effective in controlling broad-spectrum plant pathogens such as *Fusarium* spp., *Pythium* spp., and *Rhizoctonia solani* [4, 21, 39]. In addition to this point, we address the specific activity of *Burkholderia* strain O1a_RA002 to inhibition of growth of hypogeous fungi (see Table 5). On the other hand, it is less harmful to soil bacteria and symbiotic or wood-decaying fungi which develop in stem base, litter, and decorticated barks of trees. This strain- or group-specific activity appears suited for the elimination of heat- and drought-resistant hypogeous sporocarps, which are likely to remain alive and flourish over a drought spring season in the soils, after fire killed most mycorrhizal fungi directly or indirectly through the degradation of host plants [27, 33, 48]. A high abundance of antifungal bacteria in compost-treated soils, in which the levels of soil fungi are significantly lowered, is indicative for the control of soil fungi by the density-dependent manners. The consequence of this control is the decrease in the ratio of fungi to bacteria, as seen in compost-treated soils. Evidently, the results obtained from both culture-dependent and -independent analyses of soil microorganisms showed that the compost used in burned soils caused a greater impact in soil fungi than bacteria. Moreover, the compost-treated soils are potent to restore not only soil microorganisms but also soil conditions, such as organic matter, water content, and nitrogen source, being closer to those of unburned forest soils. Both biotic and abiotic properties are critical to regenerate the forest and its natural cycles. It appears that the antifungal bacteria have a role to play in the regulation of soil fungi which are related to plant pathogens and animal pathogens, so they may cause soil conditions favorable for vegetation recovery and soil macrofauna re-colonization in post-fire forest soils.

We demonstrated here that amendment of burned soils with compost is practically useful for promoting the growth of antifungal *Burkholderia* strains as well as vegetation at the shrubland stage of *L. bicolor* in Korea. The promotion of antifungal bacteria is advantageous in agriculture and forest management after a fire if they control plant pathogens and thereby cause soil conditions favorable for seedling survival and vegetation in a temperate forest as suggested by Packer and Clay [36]. A high abundance of antifungal bacteria is necessary for the inhibition of soil fungi by the density-dependent manners. Several studies suggest that some species of the genus *Burkholderia* are effective in disease suppression and growth promotion of plants [5, 9, 30, 40]. They may be helpful for the recovery of vegetation and fauna in burned soils if they can remove suspected soil pathogens such as *P. larviformis*, *M. platani*, and *M. verticillata*.

It is yet unclear, however, how the compost promotes the growth of antifungal bacteria in burned soils and what the consequence is on plant growth and soil ecosystem. In order to better understand the regulatory mechanisms on soil microbial populations and plants in post-fire forest soils, works have been going on with the use of different types of composts and soils. Starting from the year 2005 until present, the repeated, mid-term experiments in three different fire places in Kangneung, Samcheok, and Uljin within the Taebaek Mountains resulted in similar effects of

the compost on forest recovery and soil conditioning (results not presented). Those studies provide us with an insight into substantial inputs to achieve more benefits from plant–soil communities and make contribution to the post-fire strategy for recovery of the forest and maintenance of its natural cycles, e.g., nitrogen and CO_2 fixation.

As conclusion, we suggest that the beneficial effects of composting are at least twofold: one is to promote the growth of vegetation with the soil conditioning and the other is to promote the growth of antifungal strains which can control soil fungi. Both support the efforts to use compost in agriculture and forest management services upon traditional and recurrent views of compost as being more eco-friendly than chemical fertilizers and soil fumigants. This work further serves as an interesting example for practicing the post-fire forest restoration to rapidly recover soil microbes and vegetation which will function to minimize or prevent soil loss from runoff, erosion, and mineralization in the first rainfall season following the fire.

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