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Monitoring of Soil Bacterial Community and Some Inoculated Bacteria After Prescribed Fire in Microcosm

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The soil bacterial community and some inoculated bacteria were monitored to assess the microbial responses to prescribed fire in their microcosm. An acridine orange direct count of the bacteria in the unburned control soil were maintained at a relatively stable level ($2.0\sim2.7 \times 10^9$ cells/g^{-1} · soil) during the 180 day study period. The number of bacteria in the surface soil was decreased by fire, but was restored after 3 months. Inoculation of some bacteria increased the number of inoculated bacteria several times and these elevated levels lasted several months. The ratios of eubacteria detected by a fluorescent *in situ* hybridization (FISH) method to direct bacterial count were in the range of 60~80% during the study period, with the exception of some lower values at the beginning, but there were no definite differences between the burned and unburned soils or the inoculated and uninoculated soils. In the unburned control soil, the ratios of α-, β- and γ-subgroups of the proteobacteria, *Cytophaga-Flavobacterium* and other eubacteria groups to that of the entire eubacteria were 13.7, 31.7, 17.1, 16.8 and 20.8%, respectively, at time 0. The overall change on the patterns of the ratios of the 5 subgroups of eubacteria in the uninoculated burned and inoculated soils were similar to those of the unburned control soil, with the exception of some minor variations during the initial period. The proportions of each group of eubacteria became similar in the different microcosms after 6 months, which may indicate the recovery of the original soil microbial community structure after fire or the inoculation of some bacteria. The populations of *Azotobacter vinelandii*, *Bacillus megaterium* and *Pseudomonas fluorescens*, which had been inoculated to enhance the microbial activities, and monitored by FISH method, showed similar changes in the microcosms, and maintained high levels for several months.

***Key words*:** Soil bacterial community, prescribed fire, fluorescent *in situ* hybridization (FISH), inoculation

When a wildfire takes place in a forest, the temperature may increase up to 1,400°C, depending on the type and amount of biomass fuel, and damages not only aboveground organisms, like animals and plants, but also belowground organisms, including soil microorganisms. It also affects the physical and chemical characteristics of the soil, such as the structure, porosity, infiltration and nutrient content, and subsequent effects may also damage soil organisms (Neary *et al*., 1999). Soil microorganisms are very important for the maintenance of belowground systems, and various soil characteristics that support the growth of all soil organisms are deeply involved with their activities. Therefore, damage to a soil's microbial community may have many negative effects to the growth of soil organisms, including plants. Hazardous compounds, such as dioxins, polycyclic aromatic hydrocarbons and polychlorinated biphenyls, are also produced during wildfire and inhibit the soil organisms (Martínez *et al*., 2000; Gabos *et al*., 2001). Although the importance of soil microorganisms in the terrestrial ecosystem is well known, the effects of fire on the soil microbial community have not been investigated in details. There have been several reports on the fire effects on soil microbes, but these were limited to the population changes of some large taxonomic or physiological groups (Sharma, 1981; Vázquez *et al*., 1993; Harris *et al*., 1995; Acea and Carballas, 1996; Acea *et al*., 2003). Due to the differences and changes of various environmental conditions at different sites, field studies tend to show large fluctuations in the microbial populations. Furthermore, in those studies, the microbial populations were determined by plate counting, which significantly underestimate the real soil microbial numbers (Amann *et al*., 1995). Other biochemical assays, such as phospholipid fatty acid (PLFA) analysis, also can not

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show detailed changes in the soil microbial community in details (Bååth *et al.*, 1995).

Recently, a fluorescent in situ hybridization (FISH) method has been developed and applied to investigate microbial communities in environments (Hicks *et al.*, 1992; Trebesius *et al.*, 1994). The FISH method is easier and more rapid than other molecular biological techniques for the analysis of microbial community structures; however, it has mainly been used for aquatic systems (Alfreider *et al.*, 1996; Belkova *et al.*, 2003). In this study, an indigenous soil bacterial community and several bacteria were inoculated to enhance some of the microbial activities with the subsequent plant growth monitored in soil microcosms by the direct counting and FISH methods during the 180 days following the prescribed burning. The soil microcosm may ease the difficulty of studying soil bacteria due to the heterogeneous nature of the physicochemistry and changing environmental conditions in field soil ecosystems (Ellis, 2004).

Materials and Methods

Soil collection and establishment of microcosm

Surface soil (0~15 cm) and plant litter were collected separately from a temperate coniferous forest (30-year old *Pinus densiflora*) located in Kangnung, Korea. Samples were placed in large plastic bags and immediately transferred to the laboratory. The soil was sandy loam, with a pH 5.2, with an organic matter content of 3.85%, by the loss-on-ignition method. The fresh soil was sieved through a 4-mm mesh sieve, and 60 kg of the sieved soil poured into large plastic containers ($1.2 \times 0.6 \times 0.5$ m). The soil surface was covered with the plant litter (2 cm depth) and burned for 15 min by using a propane gas torch. Some of soil microcosms were inoculated with *Azotobacter vinelandii* (1.6×10^{10} cells), *Nitrosomonas europaea* (7.8×10^{9} cells), *Bacillus megaterium* (1.0×10^{9} cells) and *Pseudomonas fluorescens* (5.3×10^{9} cells) to enhance some of the microbial activities and plant growth in the soil. These bacteria had been isolated from the same soil, and were identified in the Korean Collection of Type Culture (Ahn *et al.*, 2002). All bacteria were separately cultivated, harvested by centrifugation and washed twice with distilled water. These bacteria were resuspended in 100 ml of distilled water and sprayed evenly on the surface of the soil microcosms. The microcosms (3 uninoculated unburned control, 3 uninoculated burned, 3 inoculated unburned, and 3 inoculated burned microcosms) were placed in a greenhouse in which the temperature and humidity were controlled, with automatic water spraying once in a day.

Soil sampling and direct counting of soil bacteria

The soils were sampled periodically (total 10 times) from the microcosms during the 180 day study period. From each microcosm, 3 random subsites were selected, with soils collected from the surface layer (0~3 cm depth) without disturbance of the surroundings. The soil samples from the 3 subsites were combined to make a composite sample for each microcosm. Sterile distilled water (100 ml) was added to a 10 g (wet wt) soil sample and homogenized for 45 seconds in a mechanical blender. At the same time, the soil moisture content was measured. This soil suspension was ultrasonicated for 30 min (28 KHz, 400 W) to detach the adsorbed bacteria from the mineral and organic particles. The soil suspension was diluted 10-fold in series, and the properly diluted soil suspension was fixed by neutral formalin (final conc. 2%). For the direct counting of the total bacteria, 10 μl of the fixed sample was filtered through a black polycarbonate membrane filter (Nuclepore, pore size 0.2 μm, dia. 25 mm), and stained with acridine orange (final con. 0.01%) (Hobbie *et al.*, 1977). The stained bacterial population on the membrane filter was counted under epifluorescent microscopy (Olympus BX60, Japan). The total bacterial count was averaged from the bacterial numbers obtained from 20 microscopic fields.

Table 1. The oligonucleotide probes used in this work

Probe	Probe structure (5'-3')	rRNA type, positions	Detected bacterial group
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338-355	Total Eubacteria
ALF968	GGTAAGGTTCTGCGCGTT	16S, 968-986	Alpha-Proteobacteria
BET42a	GCCTTCCCACTTCGTTT	23S, 1027-1043	Beta-Proteobacteria
GAM42a	GCCTTCCCACATCGTTT	23S, 1027-1043	Gamma-Proteobacteria
CF319a	TGGTCCGTGTCTCAGTAC	16S, 319-336	*Cytophaga-Flavobacterium*
NON338	ACTCCTACGGGAGGCAGC	23S, 1027-1043	Negative control
A.VINE488	CCCATCAATCTCTTGAAAGTTCG	16S, 488-510	*Azotobacter vinelandii*
B.MEGA172	ATCTCCCATGAAGGAGAAGATCCTAT	16S, 172-197	*Bacillus megaterium*
P.FLUO71	TAGGCTTTACTCACCCGTCCGCC	16S, 71-93	*Pseudomonas fluorescens*

Monitoring of soil microbial community and inoculated bacteria by FISH method

For the analysis of the bacterial community structure using the FISH method, 30 ml of diluted soil suspension was fixed by the addition of 10 ml of a freshly prepared 4% paraformaldehyde, with 30 μl of the fixed sample passed through 0.2 μm pore-sized polycarbonate filters. The filters were washed three times with 0.5 ml phosphate buffered saline and then sterile water to free the paraformaldehyde, and then dried in the air. The rRNA probes used were that of EUB338 for the detection of eubacteria, ALF1b for the α-subgroup of Proteobacteria, BET42a for β-proteobacteria, GAM42a for γ-proteobacteria, and CF319a for the *Cytophaga-Flavobacterium* group, which have been designed and utilized frequently for FISH analysis of environmental bacteria (Table 1) (Manz *et al*., 1992; Alfreider *et al*., 1996; Glöckner *et al*., 1999). The inoculated bacteria were monitored with the rRNA probes, A.Vine488 for *Azotobacter vinelandii*, B.MEGA172 for *Bacillus megaterium,* and P.FLUO71 for *Pseudomonas fluorescens*, which were based on the signature sequences of each species in the Ribosomal Database Project (Table 1). Oligonucleotide probes were commercially made and labeled with tetramethylrhodamine (TaKaRa, Japan). The filter was placed on a gelatin-coated slide glass, with the addition of 16 μl of hybridization solution [0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS, formamide (concentrations for EUB, ALF, BET, GAM, CF and 3 inoculated bacteria were 0, 20, 35, 35, 15 and 30%, respectively)], and then 2 μl of probe solution (conc. 25 ng/μl) (Alfreider *et al*., 1996). After mixing the solutions, each slide was placed in a hybridization chamber and incubated at 46°C for 90 min. The hybridization mixture was then removed by immersing each filter in washing buffer [20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.01% SDS, NaCl solution (concentrations for EUB, ALF, BET, GAM and CF were 0.9 M, 0.225 M, 80 mM, 80 mM and 80 mM, respectively)] at 48°C for 20 min. The slide was gently rinsed with distilled water, and air-dried. Since the β- and γ-subgroups of Proteobacteria interfere with each other, 2 μl each of non-labeled GAM42a and BET42a were used together with labeled BET and GAM probes for the detection of the β- and γ-subgroup, respectively, to avoid the interference (Manz *et al*., 1992). The procedure targeted at *B. megaterium* was almost the same, with the exception of sample pretreatment with lysozyme to rupture the cell walls prior to the hybridization step. After reaction with the lysozyme solution [37320 U, lysozyme 1 mg/100 mM Tris-HCl (pH 7.5) with 5 mM EDTA solution 1 ml] at 37°C for 10 min, the filter attached slide was washed with sterile distilled water and successively with 50, 80 and 99% ethanol, which was followed by the hybridization step. For counter staining of the total bacteria, a subset of filters was stained with 4', 6-diamidino-2-phenylindole (DAPI) alone. The DAPI and rhodamine stained bacteria on the filter were counted from over 20 microscopic fields under epifluorescent microscopy, and the cell numbers averaged. All the experiments were carried out in triplicate, and the mean values are presented.

Results and Discussion

Direct counts of soil bacteria in microcosms

Direct bacterial counts were first examined to investigate the changes in the microbial community in the soil after the fire. The number of bacteria in the unburned control soil was maintained at a relatively stable level ($2.0\sim2.7\times10^9$ cells/g · soil) during the 180 days (Fig. 1). The prescribed fire seemed to kill many of the soil bacteria in the microcosm (Walstad *et al*., 1990), and the direct count decreased to 0.8×10^9 cells/g · soil; however, some killed or injured microorganisms might be included in this figure. The bacterial numbers in the burned soil increased after the fire, but were restored to the original level after 3 months. Inoculation of some bacteria into the unburned soil increased the direct count to 7.9×10^9 cells/g · soil (Fig. 1). This elevated population was maintained for 90 days, but thereafter decreased slowly. In the inoculated burned soil, the direct count at the beginning was as not high as in the inoculated unburned soil due to the effects of the fire; however, this increased rapidly for 30 days, reaching 15.3×10^9 cells/g · soil. This increase might have been due to the utilization of the fire-killed biomass as growth substrates (Neary *et al*., 1999) and the absence of various negative interactions caused by the indigenous soil microorganisms (Atlas and Bartha, 1998). After 30 days the direct count gradually decreased, but showed a higher level than that in the uninoculated microcosm for the 180 days. In this study, direct counting showed a distinct change in the pattern of the soil bacterial populations in each microcosm. As the

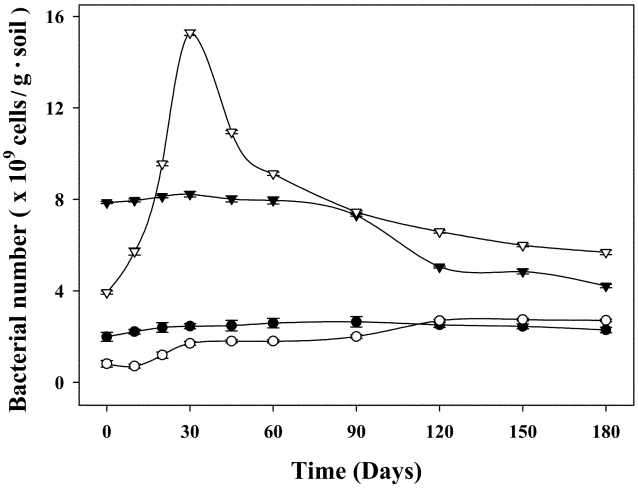

Fig. 1. Changes in the direct bacterial counts in the microcosm soils (●, Uninoculated-Unburned; ○, Uninoculated-Burned; ▼, Inoculated-Unburned; ▽, Inoculated-Burned).

direct bacterial count showed an increase during the initial 3 months, this may indicate the recovery process of the microbial community in burned soil, and also survival of the inoculated bacteria in both soils.

Changes of bacterial community after prescribed burning

In this study, the soil bacterial community in the microcosm after the prescribed fire was monitored by a fluorescent *in situ* hybridization (FISH) method after prescribed fire in the microcosm. Investigation of the microbial community structure in the environment may be important to comprehend fundamental ecosystem processes. The ratios of eubacteria detected by the FISH method to the total bacteria enumerated by direct counting were within the range of 60~80% during the study period, with the exception of some lower values at the beginning, but there were no definite differences between the burned and unburned soils or the inoculated and uninoculated soils. (Figs. 2, 3, 4, and 5). Hicks *et al.* (1992) reported 35~67% of direct counts were detectable by the FISH method using an EUB probe in a bacterioplankton community, showing ratios between 40.2~80.7% in another study on snow and a mountain lake (Alfreider *et al.*, 1996). Although there may have been some Archaea in the soil, which could not be detected with the EUB probe, these somewhat low ratios of eubacteria to direct counts were most likely due to the difference between the two enumeration techniques. Hicks *et al.* (1992) reported that about 30% of total counts were lost during the transfer from the filter to slides when using the FISH method. Probe permeability can also be another problem with the FISH analysis, which means that all bacterial cells, especially Gram-positive bacteria, bacterial endospore and bacteria with a capsule and slime layer do not easily take up the rRNA probe, including EUB, which may lower the percentage of eubacteria (Fischer *et al.*, 1995; MacGregor, 1999). The low ribosome content often found in starved cells may inhibit the FISH detection of directly counted bacteria (Glöckner *et al.*, 1999).

Changes of eubacterial community structure

In this study, the structure of the soil bacterial community was investigated with 5 different rRNA probes. Eubacteria were divided into the α-, β-, and γ-subgroups of Proteobacteria, and the *Cytophaga-Flavobacterium* group, and the others, which were not hybridized with the probes targeted for these 4 groups of eubacteria were classified as "other eubacteria". These 4 groups of bacteria are the most abundant in various natural environments, and pioneering studies on the investigation of the microbial community structure were performed with these groups of rRNA probes (Hicks *et al.*, 1992; Alfreider *et al.*, 1996). In the surface soils of the unburned uninoculated control microcosm, the ratios of α-, β- and γ-proteobacteria, the *Cytophaga-Flavobacterium* group and the other eubacteria group to that of the entire eubacteria were 13.7, 31.7, 17.1, 16.8 and 20.8%, respectively at time 0 (Fig. 2). The ratios of the α-, β-, and γ-subgroups of proteobacteria and the *Cytophaga-Flavobacterium* group ranged 9.3~33.5%, 14.1~31.7%, 10.9~20.6% and 15.3~34.5%, respectively, during the study period. In contrast, the "other eubacteria" accounted for 3.3~43.2% of the bacteria in the control surface soil. The ratios of β- and γ-proteobacteria were relatively stable, with the exception of the initial period, and those of the other groups showed somewhat higher variations. The ratios of the "other eubacteria" were smaller than those in the pine forest soil after a wildfire (Ahn *et al.*, 2002).

The ratios of each group in the surface soils of the uninoculated burned microcosm were similar to those of the unburned control, with the exception of some minor variations during the initial period (Fig. 3). Although the pre-

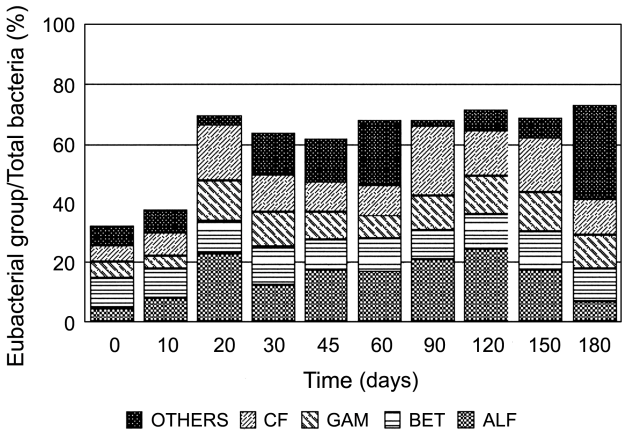

Fig. 2. Composition of the bacterial community in the uninoculated-unburned microcosm soil (ALF, α-proteobacteria; BET, β-proteobacteria; GAM, γ-proteobacteria; CF, *Cytophaga-Flavobacterium* group; OTHERS, other eubacteria).

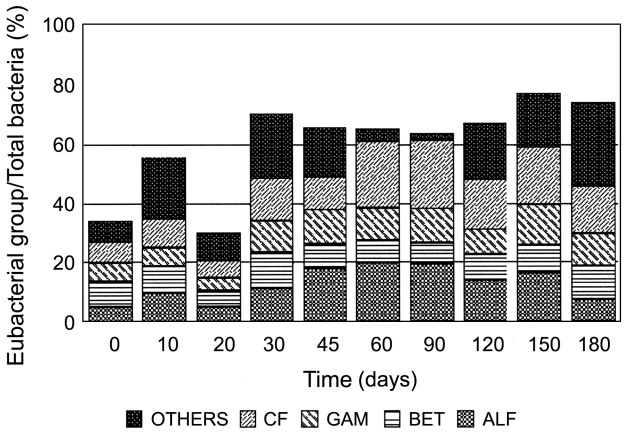

Fig. 3. Composition of the bacterial community in the uninoculated-burned microcosm soil (ALF, α-proteobacteria; BET, β-proteobacteria; GAM, γ-proteobacteria; CF, *Cytophaga-Flavobacterium* group; OTHERS, other eubacteria).

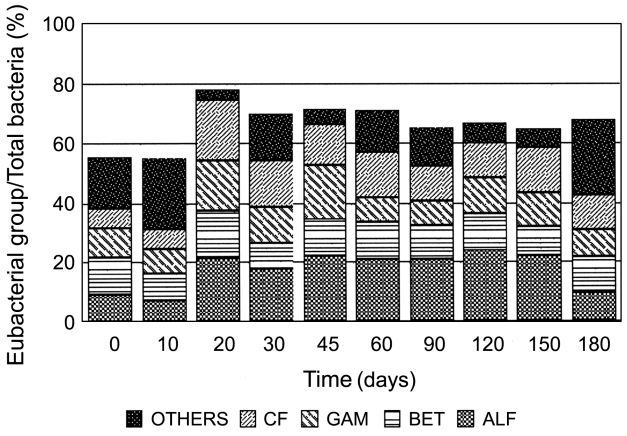

Fig. 4. Composition of the bacterial community in the inoculated-unburned microcosm soil (ALF, α-proteobacteria; BET, β-proteobacteria; GAM, γ-proteobacteria; CF, *Cytophaga-Flavobacterium* group; OTHERS, other eubacteria).

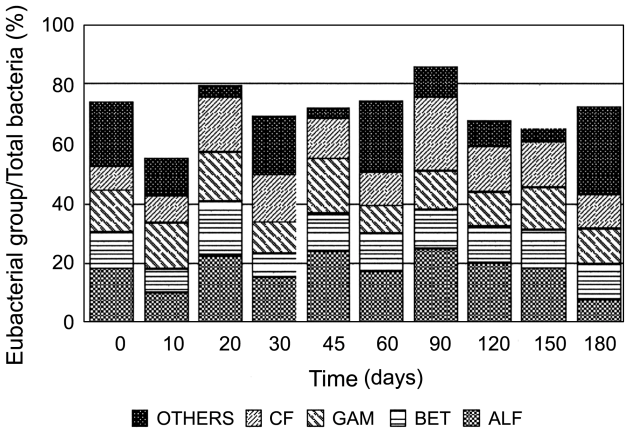

Fig. 5. Composition of the bacterial community in the inoculated-burned microcosm soil (ALF, α-proteobacteria; BET, β-proteobacteria; GAM, γ-proteobacteria; CF, *Cytophaga-Flavobacterium* group; OTHERS, other eubacteria).

scribed burning decreased the number of bacteria in the surface soil, the bacterial community structure was not significantly changed. The soil microcosm has been used in soil microbiology to ease the difficulty that arises from variable environmental conditions (Ellis, 2004); however, the heterogeneous nature of the soil physicochemistry still lead to some fluctuations, especially during the initial period, in the bacterial community structure of the microcosm soil in this study. The overall changing patterns in the ratios of the 5 subgroups of eubacteria in the inoculated microcosms were also similar to those in the uninoculated microcosms, with the exception of greater variations for the "other eubacteria" group (Figs. 4, and 5). Although the inoculation of some bacteria increased the direct counts in both unburned and burned soils (Fig. 1), it did not significantly affect the composition of the bacterial community. There might be some complex variations within each subgroup, and rRNA probes for targeting lower levels of the phylogenetic group are necessary for the investigation of the precise changes in the bacterial community structure. It has been known that the effects of fire depend on the fire intensity and duration, and the soil water and organic matter contents (Neary *et al*., 1999). Since soil environments are quite heterogeneous in terms of their texture, content and distribution of water and organic matter, the effects of fire are highly variable, with the effect on the soil microbial community even harder to examine and analyze. At the end of the study period, the proportions of each Eubacteria group became similar between the different microcosms (Figs. 2, 3, 4 and 5), which may indicate the recovery of the original structure of the soil microbial community after a fire or the inoculation of some bacteria. The high proportions of "other Eubacteria" on day 180 were most likely due to the presence of Gram-positive bacteria, such as actinomycetes, Bacilli and Clostridia (Bååth *et al*., 1995; Ahn *et al*., 2002).

Although fire decreased the total bacterial population, the bacterial community structure examined using the FISH method was not significantly disturbed by fire, and became similar in all the microcosms after several months. The FISH analysis has been known to be easier and more rapid than other molecular biological techniques for the detection of the indigenous bacteria at various taxonomic levels (Manz *et al*., 1992; Amann *et al*., 1995) and with inoculated bacteria (Martínez *et al*., 2000). Although the microscopic observation with the FISH analysis is difficult for soil samples, due to the background fluorescence and interference by soil mineral particles, this could be overcome in this study. The proportion of non-detectable bacteria from the total bacterial population using the FISH method was quite large in some cases, but the reasons for this should be revealed in detail in the near future, with the application of the FISH method for the precise analysis of the bacterial community in various environments.

Monitoring of inoculated bacteria by FISH method

Some bacteria were inoculated into the microcosm to enhance some of the microbial activities that had been disturbed by the fire, and their fates monitored using the FISH method. These inoculated bacteria had been expected to potentially stimulate the plant growth in fire damaged soil, as *Pseudomonas* and *Bacillus* are known to be typical plant growth promoting rhizobacteria, and nitrogen fixing *Azotobacter* may provide the fixed nitrogen to soil environments (Bashan, 1998). The nitrifying bacterium, *Nitrosomonas*, was inoculated to remove the excess amount of ammonium produced after a forest fire (Acea and Carballas, 1996). The number of *Azotobacter vinelandii* in the uninoculated unburned control soil was 1.0×10^7 cells/(g · soil) at time 0, and was maintained at a relatively stable level ($1.0 \sim 1.6 \times 10^7$ cells/(g · soil)) (Fig.

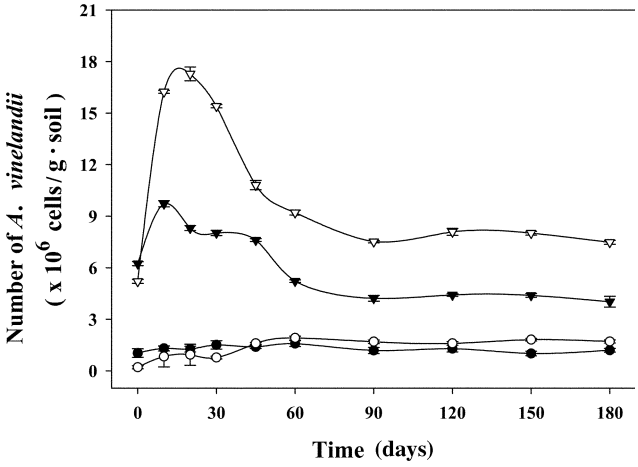

Fig. 6. Change in the number of *A. vinelandii* in the microcosm soil (●, Uninoculated-Unburned; ○, Uninoculated-Burned; ▼, Inoculated-Unburned; ▽, Inoculated-Burned).

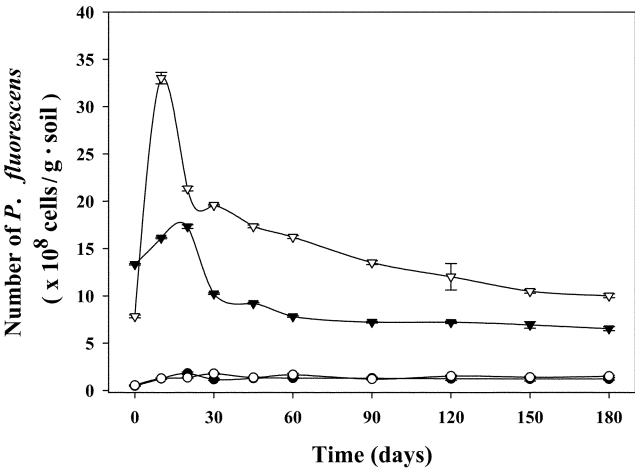

Fig. 7. Changes in the number of *P. fluorescens* in the microcosm soil (●, Uninoculated-Unburned; ○, Uninoculated-Burned; ▼, Inoculated-Unburned; ▽, Inoculated-Burned).

6). The rRNA probes targeting each inoculated species could detect the same species in the soil environments; therefore, the indigenous population of *A. vinelandii* showed a certain level in the uninoculated control soil. The number of *A. vinelandii* decreased to 0.2×10^7 cells/(g · soil) due to the prescribed fire, but increased slowly, and maintained a stable number after day 45. Inoculation of *A. vinelandii* rapidly increased the numbers until days 10 to 20. The elevated numbers decreased rapidly until day 60, and thereafter, were maintained at relatively steady figures. The population of *A. vinelandii* was higher in the burned than unburned soil during the study period, which might have resulted from the utilization of fire-killed biomass and fewer negative interactions in the burned soil (Atlas and Bartha, 1998; Neary *et al.*, 1999). *P. fluorescens* and *B. megaterium* also showed a similar pattern to that of *A. vinelandii* (Figs. 8, 9). The population

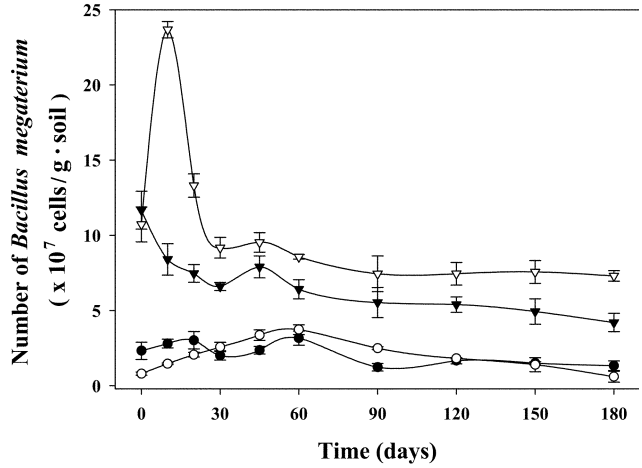

Fig. 8. Changes in the number of *B.* in the microcosm soil (●, Uninoculated-Unburned; ○, Uninoculated-Burned; ▼, Inoculated-Unburned; ▽, Inoculated-Burned).

of *Nitrosomonas europaea* could not be monitored due to the difficulty of making a species-specific rRNA probe.

All three bacteria maintained the higher population levels in the inoculated soils compared to the uninoculated soils during the 6 months, which indicates that inoculated microorganisms can multiply and survive during a quite long periods in soil environments. Our results also showed that the number of the inoculated bacteria was maintained at a very high level at less than 2 months; therefore, additional inoculation may be necessary to maintain the elevated population and activities of the inoculated microorganism.

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