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Development of Substrate Induced Respiration (SIR) Method Combined with Selective Inhibition for Estimating Fungal and Bacterial Biomass in Humic Andosols

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Abstract : A substrate-induced respiration (SIR) method combined with selective inhibition for estimating the fungal and bacterial biomass of volcanic ash of Kanto Loam type (Humic Andosols) was developed. The optimum time for CO_2 measurement was 2-4 hr after glucose application and the amount to be applied was 2000 ppm on a soil dry weight basis. CO_2 evolution from the soil correlated with the amount of microbial biomass carbon estimated by the chloroform fumigation extraction (CFE) method and a linear relationship, biomass C (μg g^{-1} dry soil) = 81.9 x CO_2 (μl g^{-1} dry soil hr^{-1}), was obtained between them. Cycloheximide (1000-8000 ppm) and chloramphenicol (500-2000 ppm) effectively inhibited SIR but streptomycin (500-2000 ppm) did not. The combination of 2000 ppm cycloheximide and 1000 ppm chloramphenicol showed the best additivity and enabled the measurement of the proportions of fungal and bacterial biomass. The SIR method combined with the selective inhibition technique was applied to soil samples from a maize field. The total microbial biomass was higher in the soil where green manure had been incorporated. The fungal-to-bacterial ratio increased from 1.1 at the time of maize sowing to 1.4 at the time of maize harvest.

Key words : Andosols, Chloramphenicol, Cycloheximide, Green manure, Microbial biomass, Selective inhibition, Substrate induced respiration.

Soil microbial biomass affects soil physical properties (Lynch and Bragg, 1985), nutrient cycles (Paul and Clark, 1989), soil ecosystem (Coleman and Crossley, 1996) and plant growth in crop production. In order to establish crop management practices that promote the fertility and productivity of soils, we need to examine the influence of different management practices on the soil microbial biomass and also to distinguish fungal and bacterial populations, because these two groups differently influence soil structure (Tisdall and Oades, 1982) and nutrient cycling (Nakas and Klein, 1980; Hendrix et al., 1986; Beare et al., 1990).

Several methods have been used to measure microbial biomass in soil. Chloroform fumigation and incubation (CFI) method (Jenkinson and Powlson, 1976) and chloroform fumigation and extraction (CFE) method (Vance et al., 1987) have been used for estimating total microbial biomass. Microscopic methods have been used to estimate the fungal and bacterial biomass separately (Jones and Mollison, 1948; Jenkinson et al., 1976; Brookes et al., 1986), but such a direct approach for differentiating bacterial and fungal populations within the total microbial community requires much time and high manipulative skills. Although the measurement of cellular components, such as fatty acids (Zelles et al., 1992) and ergosterol (Grant and West, 1986), enables the quantification of bacterial and fungal biomass, there is a need to exclude effectively the substantial amount of these substances in extracellular forms (Durska and Kaszubiak, 1983). Substrate induced respiration (SIR) method, on the other hand, is far simpler, and, when successfully combined with the use of selective inhibitors, it enables the measurement of total, fungal, and bacterial biomass in a short time (less than 6 hr).

SIR method was originally introduced by Anderson and Domsch (1973). In this method, the microbial population in soil is activated by the addition of a readily decomposable respiratory substrate (usually glucose), and the resulting initial maximal respiration is used for estimation of microbial biomass. The measurement of fungal and bacterial biomass by using selective inhibitors is based on the following assumptions: The fungal and bacterial populations show a similar respiratory response to glucose, and the fungal-to-bacterial ratio in the inhibitor-sensitive component of the biomass is equal to that in the insensitive component. Although this technique is already used widely in soil microbial studies, it does have some limitations. Inhibitors may affect a non-target population, e.g., some fungal inhibitors suppress

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Abbreviations : CFE, chloroform fumigation and extraction; CFU, colony forming unit; SIR, substrate induced respiration.

Table 1. Three treatments with or without green manure from which sample soil was collected.

Treatment	2001			2002		
	Green manure	Growth period	Above-ground biomass incorporated (g m^{-2})	Green manure	Growth period	Above-ground biomass incorporated (g m^{-2})
GM1	Bristle oat (*Avena strigosa*)	24 April－4 July	665	White mustard (*Brassica alba*)	10 April－13 June	58
GM2	Grass sorghum (*Sorghum bicolor*)	24 April－4 July	768	Bristle oat (*Avena strigosa*)	10 April－13 June	263
Control	－	－	0	－	－	0

or stimulate bacterial growth (Johnson et al., 1996; Velvis, 1997; Lin and Brookes, 1999a). Soil texture may also affect the apparent resistance to inhibitors (Coleman and Crossley, 1996). Therefore, determining appropriate inhibitors and their concentrations is the first essential step for application of this method to sampled soil.

The purpose of this experiment was to develop a SIR method combined with selective inhibition technique for estimating total microbial, fungal and bacterial biomass in volcanic ashes of Kanto Loam type (Humic Andosols).

Materials and Methods

1. Sample soil

Soil (Humic Ansosols) used in this experiment was sampled in 2002 from a field at the University Farm of the University of Tokyo, Japan, where an experiment on green manuring had been conducted since 2001. Three treatments, two with green manure differing in quality and amount of incorporated biomass and one without green manure (control), were arranged in a 3 × 3 Latin square, each plot being 4.6 m x 14.0 m (Table 1). In 2002, maize (Zea mays L., cv. KD670) was sown on 19 July, one month after green manure incorporation, and harvested on 23 September. Neither fertilizer nor pesticide was applied throughout the experimental period. Soil at the depth of 1-11 cm was taken with a boring sampler (diameter of 4 cm) from 4 - 8 randomly chosen spots in each plot and combined into a single sample.

2. Substrate induced respiration (SIR)

The optimum glucose concentration and measurement time were determined. Soil samples, collected just before green manure incorporation, were sieved with a 2 mm mesh and stored at 5 °C in aerated plastic bags for a maximum of 3 weeks. The samples were kept at 22 °C for 24 hr before assay. Fresh soil equivalent to 10 g dry soil was weighed into a plastic beaker and supplemented with 10 mg (1000 ppm), 20 mg (2000 ppm), 40 mg (4000 ppm), or 80 mg (8000 ppm) glucose, which had been mixed with 50 mg talc in advance to achieve even distribution in the soil. After thorough mixing, the soil was transferred to a 200 ml Erlenmeyer flask, sealed with a silicon rubber stopper with an inlet and an outlet with stopcocks, and statically incubated at 22 °C in a closed system. Every hour for a period of 5 hr, the inlet and outlet were connected to an infrared CO_2 analyzer (Iijima Electronics, LX-720) with flexible tubes. The air inside the flask was circulated with the flow rate of 250 ml min^{-1} and CO_2 concentration (ppm) was recorded.

In order to obtain an analytical curve to convert SIR values into microbial biomass C, we measured microbial biomass C in the soil using the CFE method. Prior to fumigation, fresh soil samples were incubated at 25 °C for 7 d. An unfumigated control, equivalent to 25 g dry soil, was extracted with 100 ml 0.5 M K_2SO_4 for 30 min on a reciprocal shaker at the rate of 200 min^{-1} and the resulting mixture was filtered. Another 25 g soil was fumigated for 24 hr at 25 °C with ethanol-free $CHCl_3$ and extracted. The amount of C in extract solutions was measured using a total organic carbon analyzer (Shimadzu, TOC-5000A) and microbial biomass C was calculated as 2.06 E*c* (J.S.S.M., 1992), where *Ec* = (C evolved from fumigated soil) - (C evolved from unfumigated soil).

3. Selective inhibition

Soil was collected at the time of maize sowing, sieved, and stored, as described above. It was then mixed with 2000 ppm glucose, 50 mg talc and inhibitors at various concentrations and incubated. CO_2 evolved between 2 and 4 hr of incubation was measured and the inhibitory effect of each inhibitor was calculated. Inhibitors tested were cycloheximide for inhibition of fungi and streptomycin sulfate, chloramphenicol, kanamycin sulfate, tetracycline hydrochloride and ampicillin sodium for suppression of bacteria.

One problem in the selective inhibition technique is the possible non-target effects of inhibitors, i.e. bactericide that inhibits fungal growth or fungicide that inhibits bacterial growth. In this study, cycloheximide (fungicide) and chloramphenicol

(bactericide) were chosen as the biocides, as described later. Therefore, the combination of their concentrations causing the least non-target inhibition was determined by calculating the additivity ratio of combinations of cycloheximide and chloramphenicol at various concentrations. The additivity ratio (AR) was calculated from the SIR of intact soil (R) and that of soil with chloramphenicol (R_B), cycloheximide (R_F), and both chloramphenicol and cycloheximide (R_{BF}). According to Beare et al. (1990),

$$AR = (I_B + I_F)/I_{BF},$$

where I_B (= [R - R_B]*100), I_F (= [R - R_F]*100) and I_{BF} (= [R - R_{BF}]*100) are the percentages of inhibition caused by chloramphenicol, cycloheximide and both, respectively. The additivity ratio should equal 1 if non-target inhibition does not occur. An additivity ratio greater than 1 indicates non-target inhibition and that smaller than 1 non-target stimulation (Beare et al., 1990). Concentrations of inhibitors tested were 2000 ppm, 4000 ppm and 8000 ppm for cycloheximide and 1000 ppm and 2000 ppm for chloramphenicol.

Bacterial and fungal contributions to the total biomass were calculated from SIR with the optimal concentrations of chloramphenicol and cycloheximide determined above. The equations used were,

Bacterial SIR (% of total SIR)
$= [\{(R - R_B)+(R_F - R_{BF})\}/2]/(R - R_{BF})*100$
Fungal SIR (% of total SIR)
$= [\{(R - R_F)+(R_B - R_{BF})\}/2]/(R - R_{BF})*100.$

$(R - R_B)$ and $(R_F - R_{BF})$ represent bacterial respiration. $(R - R_F)$ and $(R_B - R_{BF})$ represent fungal respiration and $(R - R_{BF})$ represents respiration inhibited by a mixture of chloramphenicol and cycloheximide.

The inhibitory effects of chloramphenicol and cycloheximide on bacteria and fungi, respectively, were confirmed by dilution plate culture (J.S.S.M., 1992). Soil was taken from one of the control plots after maize sowing. Colony forming units (CFU) of fungi was measured on rose-bengal agar and that of bacteria on YG agar, with 2000 ppm glucose supplemented with chloramphenicol (1000 ppm), streptomycin (1000 ppm), or cycloheximide (2000 ppm). Concentrations of glucose and inhibitors were based on the wet weights of the culture media. After incubating the plates at 25 °C for 5 d, CFU on three plates at adequate dilution levels were counted.

Results

1. Optimum time of CO_2 measurement and glucose concentration

The pattern of microbial respiration was observed for 5 hr after incubation with glucose to determine the time when evolved CO_2 best represents the size of the original microbial population in soil. The rate of CO_2 release decreased during the first 2 hr and was fairly stable from 2 to 4 hr (Fig. 1). After 4 hr of incubation, the rate of CO_2 release increased in some samples.

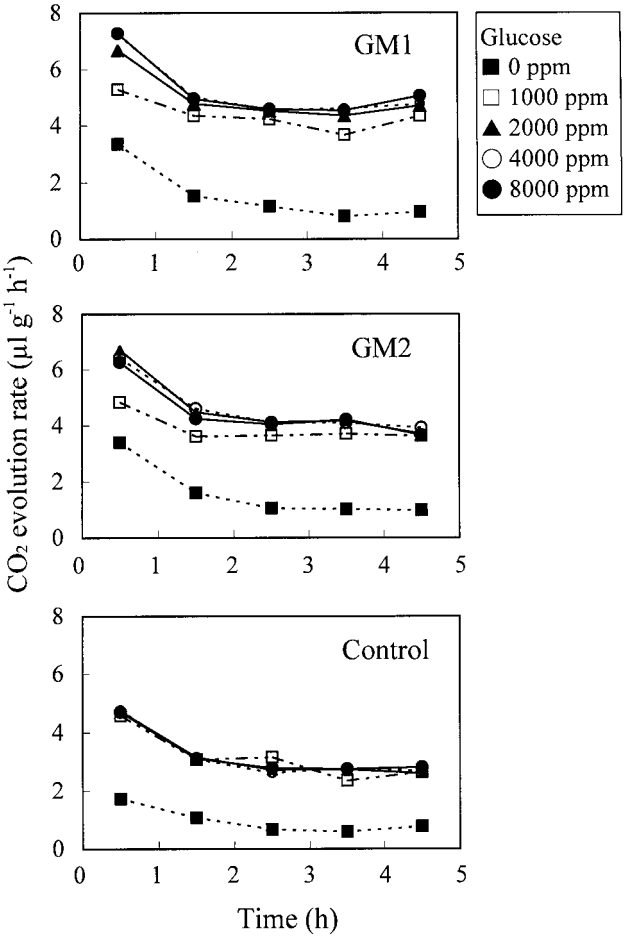

Fig. 1. Changes in the rate of CO_2 evolution from the three types of soil collected from the plots with (GM1, GM2) or without (Control) green manure during incubation with glucose at various concentrations.
GM1, GM2, Control; see Table 1.

Measurement should start after the initial declining of CO_2 release ceased (Anderson and Domsch, 1978), and end before the increase of CO_2 release, which indicates a new biomass synthesis (Anderson and Domsch, 1978; Beare et al., 1990). In this respect, the best time for measurement was between 2 and 4 hr.

Glucose supplementation stimulated CO_2 release from the soil, indicating that soil microorganisms were activated by the addition of respiratory substrate (Fig. 1). The highest rate of respiration was attained by applying 2000 - 8000 ppm glucose to the soil from green manure plots (GM1, GM2) and 1000 - 8000 ppm glucose to the control soil. The minimum concentration of glucose giving the maximal respiratory response (Anderson and Domsch, 1978; Beare et al., 1990) was 2000 ppm, and this was adopted for further analysis.

Fig. 2 shows the correlation of microbial respiration (CO_2 revolution rate) measured by the SIR method (22 °C, 2-4 hr, 2000 ppm glucose) with the microbial biomass C estimated by the CFE method. The

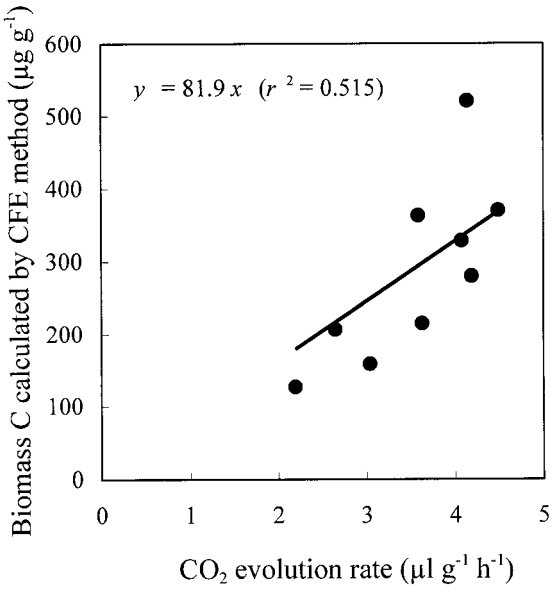

Fig. 2. Relationship between SIR and biomass C estimated by the CFE method.

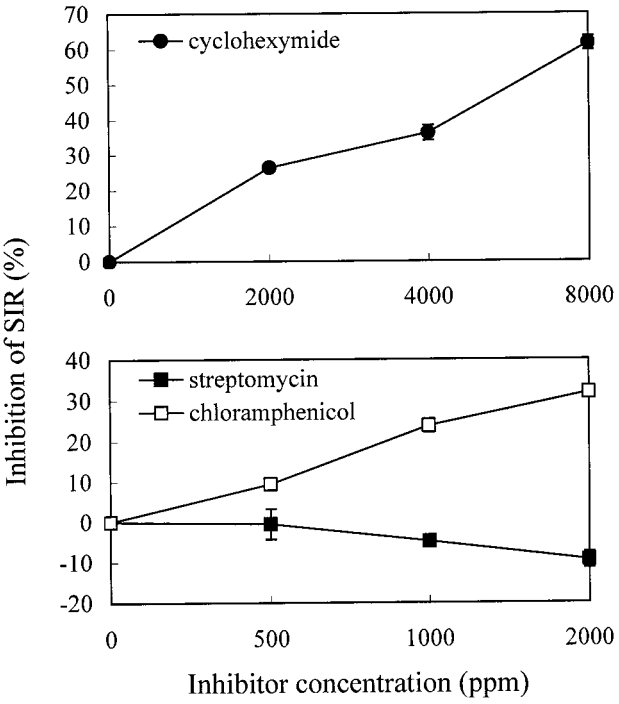

Fig. 3. Inhibition of SIR by cycloheximide, streptomycin, and chloramphenicol.
Bars indicate standard errors (n=3).

microbial respiration showed a significant positive correlation with the microbial biomass C ($p<0.05$). The correlation coefficient was 0.72 and the regression equation was $y = 81.9\ x$, where x = SIR (μl CO_2 g^{-1} dry soil hr^{-1}) and y = biomass C measured by CFE (μg biomass C g^{-1} dry soil). This equation was used to convert SIR values into microbial biomass C.

2. Optimum concentrations of selective inhibitors

The optimum concentrations of cycloheximide and streptomycin, which are the most widely used for selective inhibition of fungi and bacteria, respectively, were tested. Cycloheximide at 2000, 4000 and 8000 ppm inhibited the microbial respiration by 26.4, 36.2 and 61.5%, respectively (Fig. 3a), but streptomycin at 500, 1000 and 2000 ppm stimulated the microbial respiration by 0.4, 4.9 and 9.5%, respectively (Fig. 3b). It was clear that streptomycin was unsuitable for selective inhibition of microbes in this type of soil. Therefore, four other antibiotics known to inhibit prokaryotes, kanamycin, tetracycline, ampicillin, and chloramphenicol, were tested in search of an effective inhibitor for bacteria. Among the four inhibitors tested, chloramphenicol was the only bactericide showing dose-dependent microbial inhibition (Fig. 3b). It reduced respiration by 9.4, 23.4, and 31.6% at 500, 1000 and 2000 ppm, respectively. Kanamycin, tetracycline, and ampicillin had slight stimulating effects like streptomycin at all concentrations tested (data are not shown).

The additivity ratio was closest to 1 when 1000 ppm chloramphenicol was combined with 2000 ppm cycloheximide (Table 2). When the two inhibitors were applied at higher concentrations, the additivity ratio exceeded 1 by more than 5%, indicating a significant non-target inhibition by chloramphenicol or cycloheximide. Therefore, the combination of 1000 ppm chloramphenicol and 2000 ppm cycloheximide was selected as the optimum concentrations for target-specific inhibition.

Table 2. Inhibition of SIR (%) by chloramphenicol and cycloheximide at various concentrations and additivity ratio (AR).

Cycloheximide concentration (ppm)	Chloramphenicol concentration (ppm)		
	0	1000	2000
0	0	23.5	33.7
2000	27.9	53.1 AR = 0.97	56.5 AR = 1.09
4000	37.3	56.9 AR = 1.07	61.3 AR = 1.16
8000	55.8	62.8 AR = 1.26	63.8 AR= 1.40

$AR = (I_{cyc} + I_{chl})/I_{cyc+chl}$.
Means of 6 data.

Table 3 shows the effect of the inhibitors on the growth of fungi and bacteria in the agar-plate culture. It was obvious that cycloheximide and chloramphenicol were effective in inhibiting fungal

Table 3. Effects of inhibitors on colony forming unit (CFU) of fungi and bacteria determined by the dilution plate method.

	Control (no inhibitor)	Inhibitor		
		Chloramphenicol (1000 ppm)	Streptomycin (1000 ppm)	Cycloheximide (2000 ppm)
Fungi (x 10^4)	6.5 ± 0.6 (100)	12.9 ± 0.8 (198)	10.9 ± 0.3 (168)	0.7 ± 0.1 (11)
Bacteria (x 10^6)	13.5 ± 0.5 (100)	0.0 ± 0.0 (0)	0.5 ± 0.2 (4)	14.7 ± 1.1 (109)

CFU (g^{-1} dry soil) ± s.d. (n=3) and % to the control (in parentheses).

and bacterial growth, respectively, even in this type of soil. Although cycloheximide had almost no effect on bacterial CFU, both chloramphenicol and streptomycin increased fungal CFU.

3. Effect of green manure application on microbial biomass

The total, fungal, and bacterial biomass in the soils from GM1, GM2 and control plots in Table 1 were estimated by the SIR method under the conditions determined above, i.e. the evolved CO_2 was measured between 2-4 hr after application of 2000 ppm glucose in combination with 2000 ppm cycloheximide and 1000 ppm chloramphenicol at 22 °C . The total biomass was higher in GM1 and GM2 with green manure compared to the control (Fig. 4). This was in accordance with the well-known fact that organic matter incorporation enhances the microbial biomass in soil. Although the fungal-to-bacterial ratio was not significantly different among the three plots, the average of the ratios in the three plots (± standard deviation) increased from the time of maize sowing (1.10 ± 0.30) to the time of maize harvest (1.43 ± 0.21) ($p < 0.05$ by Student's *t*-test).

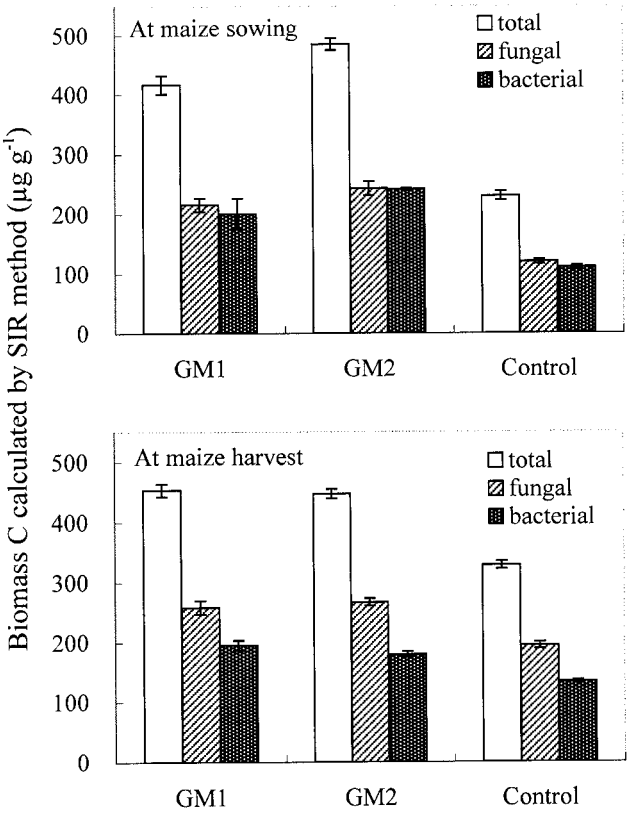

Fig. 4. Total, fungal, and bacterial biomass in the three plots (GM1, GM2, Control) determined by the SIR method combined with selective inhibition.
GM1, GM2, Control; see Table 1.
Bars indicate standard errors (n=3).

Discussion

Although this experiment was undertaken using simple equipment, the rate of CO_2 evolution from the soil was significantly correlated with biomass C calculated by the CFE method, which is one of standard methods for measuring soil microbial biomass. The procedure developed here will be useful to examine microbial biomass in Andosols, which is known to contain a large amount of humus (Wada, 1985). The curve of CO_2 release during 5 hr after glucose supplementation was similar to the type II curve observed by Anderson and Domsch (1978). Although it is not known why the SIR rate declines in the early hours of incubation, this pattern is known to appear in the soils rich in organic matter, such as forest soils (Anderson and Domsch, 1978).

The selective inhibition method is useful for estimating the proportion of fungal to bacterial biomass in the active portion of soil microbial biomass. However, it should be used with some care because there may be non-target inhibition by inhibitors (Velvis, 1997), inhibitor degradation by resistant populations, inhibitor inactivation by soil matrix, and changes in microbial competition (Parkinson et al., 1971). Hence, the search of inhibitors and their concentrations most appropriate for sample soil is an important task. Inhibitors most widely used for selective inhibition in the SIR method are cycloheximide and streptomycin

(Anderson and Domsch, 1975; Beare et al., 1990; Wardle and Parkinson, 1990a; Alphei et al., 1995; Lin and Brookes, 1999b; Henriksen and Breland, 2002). In this research, cycloheximide was effective in reducing microbial respiration, possibly because of the inhibition of fungal respiration. However, streptomycin stimulated microbial respiration, as had been reported in several previous studies (Johnson et al., 1996; Lin and Brookes, 1999a). It can be postulated that decline in bacterial respiration by streptomycin was canceled by the increase in fungal respiration (Scheu and Parkinson, 1994). On the other hand, chloramphenicol caused consistent inhibition of respiration. The combination of 1000 ppm chloramphenicol and 2000 ppm cycloheximide was proved to be the best for discriminating bacterial and fungal biomass, because the additivity ratio was nearly equal to 1 (Table 2), indicating that non-target effects were negligible at these concentrations. The non-target effects of cycloheximide and chloramphenicol in dilution plate culture (Table 3) were not reflected in SIR inhibition at the same concentrations (Table 2). Because the incubation time of the plates was much longer than that for SIR method, antibiotics might differently affect non-target organisms.

It should be noted that the inhibitors used for the selective inhibition inhibited about a half of the respiration of total biomass in soil. Increasing concentrations of cycloheximide and chloramphenicol resulted in more than 60% respiratory inhibition, but optimal additivity was achieved when the reduction was near 50% (Table 2). These levels of inhibition are consistent with those demonstrated by Anderson and Domsch (1975), West (1986), Wardle and Parkinson (1990b) and Johnson et al. (1996). If the community structure greatly differs between inhibited and uninhibited parts of soil microbes, this method may give erroneous results. Therefore, selective inhibition is suitable for examining microbial changes within the same field rather than to compare various soils from different fields. The method developed here was useful for detecting the increase in total microbial biomass caused by the addition of organic matter, green manure residues, to the soil. It also revealed a fungal dominance in this soil, which tended to be intensified during maize growth. Fungal dominance in agricultural and forest soils has been reported by many selective inhibition studies (Anderson and Domsch, 1975; Wardle and Parkinson, 1990a; Johnson et al., 1996; Lin and Brookes, 1999a). Although the selective inhibition should be confirmed by other methods, such as direct microscopy or cellular component analysis, it is a simple method useful for crop scientists to study soil microbial dynamics.

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