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Microbial community structure in a silty clay loam soil after fumigation with three broad spectrum fungicides

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The short-term effect of three broad spectrum fungicides on microbial activity, microbial biomass, soil ergosterol content, and phospholipid fatty acid (PLFA) profiles was studied. A silty clay loam soil was treated separately with captan, chlorothalonil and carbendazim at three different dosages of each fungicide. Chlorothalonil and carbendazim significantly altered soil microbial activity. However, changes in soil microbial biomass were only observed in soil treated with higher dosages of these fungicides. All dosages of fungicides significantly decreased fungal biomass as estimated by soil ergosterol content. PLFA analysis indicated that there was a shift in PLFA pattern. Higher dosages of all three fungicides decreased a straight-chain PLFA 22:0. In addition, soil treated with carbendazim increased cyclopropyl fatty acids. Compared to untreated soil, higher dosages of both captan and chlorothalonil affected PLFA 10Me 16:0, indicating that these fungicides can reduce actinomycetes population. Finally, our results suggest that application of both captan and chlorothalonil decreased Gram-positive to Gram-negative ratio.

Keywords: Soil microbial; fungicides; microbial biomass; microbial activity; fungal biomass; microbial community structure.

Introduction

The intensive use of fungicides in agricultural systems has become a matter of environmental concern, potentially because of the effect of these biocides on non-target microorganims.[1] In recent years, the effect of agrochemicals on soil microbial populations has been studied extensively. However, compared with studies about environmental effects of herbicides or insecticides, there are few reports about the ecotoxicological effect of fungicides on soil microbial communities.[2–4]

Several authors observed that fungicide applications killed or inhibited the activity of a certain group of microorganims.[5] The fungicides captan, chlorothalonil and carbendazim are considered to be non-selective and are used to control a broad spectrum of plant diseases, principally in natural and artificial grasslands. Chen and Edwards[6] reported that both captan and chlorothalonil suppressed the peak of soil microbial respiration. Carbendazim, is known to disrupt mycorrhiza functioning[7] and inhibit bacterial and yeast processes.[8] Sousa et al.[9] demonstrated that carbendazim application produce changes on soil phosphatase activity. However, the effect of these fungicides on soil microbial community structure has not been established.

Many toxicity tests have been developed to assess the impact of biocides application to soils. Total microbial activity is an important parameter of ecological tests. The potential of fluorescein diacetate (FDA) hydrolysis as a measure of whole microbial activity has been recognized by many authors and used on a wide range of samples.[10,11] Ergosterol is endogenous almost exclusively to fungi and so may be a useful index to estimate content of the living soil fungal biomass.[12] It is widely accepted that phospholipids fatty acids (PLFA) analysis is a powerful and responsive tool for microbial community composition description.[13] Environmental disturbances, management practices, and plant natural invasions can cause rapid changes in soil microbial community structure, as indicated by changes in soil PLFA profiles.[14,15,16] Although some works on the effect of fungicides on soil microbial biomass and chemical parameters have been conducted, little is known about their effects on soil microbial profiles and diversity. The purpose of this paper is to examine captan, chlorothalonil, and carbendazim effects on microbial activity, fungal biomass, and microbial community structure.

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Materials and methods

In November 2006, the 0–10 depth layer of natural grassland located at the National University of Córdoba (Córdoba city, Argentina) was sampled. The climate regime of this region is semiarid with annual precipitations between 700–800 mm, and moderate temperatures (16–17°C of annual overage). The soil is a silty clay loam with a mean particle size distribution of 16% sand, 55.2% silt, and 28.8% clay. The main soil chemical characteristics were: 8.5% organic C, 0.42% total N, pH 7.1, and 1.8 of C/N relation. Five-hundred grams of sieved soil was placed in plastic containers, and constant moisture content was maintained by the weekly addition of sterilized water to restore the initial weight. Commercial grades of the fungicides captan (Captan, 80% wettable powder), chlorothalonil (Daconil 72 F) and carbendazim (Hon Glex, garden formulation) were applied by spraying appropriately diluted solutions at recommended field rates (0.125 g captan Kg^{-1}, a.i.; 0.054 g chlorothalonil Kg^{-1}, a.i; 0.45 mg carbendazim Kg^{-1}, a.i.), 10 times these rates (1.25 g captan Kg^{-1}, a.i.; 0.54 g chlorothalonil Kg^{-1}, a.i; 4.5 mg carbendazim Kg^{-1}, a.i.), and 100 times these rates (12.5 g captan Kg^{-1}, a.i.; 5.4 g chlorothalonil Kg^{-1}, a.i; 45 mg carbendazim Kg^{-1}, a.i.). Recommended field rates were calculated considering a soil layer of 2 cm and soil bulk density of 1.36 cm^{-3}. There were three replicate containers for each fungicide treatment. The controls soils were treated with equal amounts of distillated water. At 3, 11, 30, and 65 days after fungicide treatment (DAFT), 100 g of soil subsamples were taken from plastic containers to assess fungicide effect at temporal intervals. Soil subsamples were stored at 4°C and dark until laboratory analysis.

Microbial biomass C was determined using the chloroform fumigation-incubation technique according to Jenkinson and Powlson.[17] Microbial biomass C was calculated as the difference in CO_2-C between fumigated and unfumigated samples.[18] Microbial activity was measured by hydrolysis of fluorescein diacetate (FDA) using a modified procedure of Adam and Duncan.[19] Two grams of soil were placed in a conical flask with 15 mL of 60 mM potassium phosphate buffer pH 7.6. Stock solution (0.2 mL) was added to start the reaction. The flasks were placed in an orbital incubator at 30°C for 20min, 100 rpm. Once removed from the incubator, 15 mL of the chloroform/methanol (2:1 v/v) was added immediately to terminate the reaction. Stoppers were replaced on the flasks and the contents shaken thoroughly by hand. The contents of the conical flasks were then transferred to 50 mL centrifuge tubes and centrifuged at 2000 rpm for 3 min. The supernatant from each sample was then filtered into 50 mL conical flasks and the filtrates measured at 490 nm on a spectrophotometer.

Fungal biomass was estimated by soil ergosterol content according to microwave-assisted protocol.[20] Each soil sample was treated with 2 mL of MeOH and 0.5 mL of 2M NaOH and then irradiated at 2450 MHz and 900 W maximum output. Ergosterol was extracted three times with hexane, filtered, and evaporated to dryness under nitrogen flow. Samples were reconstituted with 200 μL of methanol and analyzed with a Perkin Elmer HPLC equipped with an UV detector and reverse-phase column (Microsorb-MV C18, Varian) at 282 nm.

Microbial community composition was measured using PLFA analysis described previously.[13,21] Only PLFAs on the three fungicides at 65 DAFT were measured. Lipids were extracted from 10 g of each soil samples using a single phase chloroform:methanol:citrate buffer (1:2:0.8 v/v/v). After extraction the lipids were separated into neutral lipids, glycolipids, and phospholipids on a silicic acid column. The phospholipids were methylated and separated on a gas chromatograph equipped with an Elite–5 capillary column and flame ionization detector. All solvents and chemicals used were of analytical grade. Identification of the PLFAs was by comparison of retention time and equivalent chain length with known standards (Bacterial Acid Methyl Esters CP Mix, Supelco USA) and confirmed by gas chromatography mass spectrometry (GC-MS). The fatty acid nomenclature used is as follows; total number of carbon atoms:number of double bonds, followed by the position (ω) of the double bond from the methyl end of the molecule. Cis and trans configurations are indicated by c and t, respectively. Anteiso- and isobranching are designated by the prefix a or i. therefore, 10Me is a methyl group on the 10th carbon atom from the carboxyl end of the molecule. Cy indicates cyclopropane fatty acids. Br indicates a branched fatty acid with unknown branching configuration.

Individual PLFAs were used as biomarkers of Gram-negative bacteria, Gram-positive bacteria, and actinomycetes. The PLFAs chosen have been used for this purpose in other studies.[16,22,23] The PLFAs selected were: Gram-negative bacteria, 16:1ω9c, cy17:0, 18:1ω9c, 18:1ω7c and cy19:0; Gram-positive bacteria, i15:0, a15:0, i16:0, i17:0; actinomycetes, 10Me 16:0. PLFA 18:2 represents a coeluting mixture of isomers 18:2ω6,9 and 18:2ω9,12. Saturated PLFAs were 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0. Unsaturated PLFAs were 16:1ω9c, 18:1ω9c, 18:1ω7c. The ratio cyclopropyl fatty acid to monoenoic precursors was calculated as follows: (cy17:0+cy19:0)/(16:1ω9c+18:1ω7c).

Fungicide effects on microbial biomass, fungal biomass, microbial activity, and individual PLFAs were determined at each sampling point by a one-way analysis of variance(ANOVA) (INFOSTAT/Professional 2005p.1), and multiple comparisons within the ANOVAs were done with least significant difference (LSD) post hoc test. Means and SE reported in tables and figures are untransformed data. The principal component analyses (PCA) was done using a correlation matrix with data standardized to unit variance. PCA of the results of PLFA biomarkers were performed to elucidate major variation in fungicide treatments. PLFAs that only appear in a few samples are unreliably not reported because they have values near the detection limit.

Table 1. Microbial activity (μg fluorescein g^{-1} soil) in soil treated with different dosages of captan, chlorothalonil, and carbendazim.[1]

	Days after fungicide treatment (DAFT)			
Treatment (mg Kg^{-1})	*3*	*11*	*30*	*65*
Control	7.11 ± 0.7	11.11 ± 5.0	11.52 ± 1.3	10.30 ± 0.7
3.5 captan	9.20 ± 2.5**	14.89 ± 2.6**	13.06 ± 1.6	13.55 ± 2.0 +
35 captan	7.38 ± 0.8	13.04 ± 2.3	9.15 ± 2.7**	11.42 ± 1.2
350 captan	6.44 ± 0.6	9.79 ± 0.7	11.10 ± 0.8	9.90 ± 0.5
2.5 chlorothalonil	8.46 ± 1.0	14.24 ± 0.1	13.10 ± 0.4	12.58 ± 1.4*
25 chlorothalonil	7.09 ± 1.5	14.75 ± 0.2	12.47 ± 1.6	10.71 ± 0.3
250 chlorothalonil	9.55 ± 0.8*	12.52 ± 0.7	7.09 ± 0.5*	7.91 ± 0.7*
1.7 carbendazim	8.02 ± 1.1	8.39 ± 0.4	18.42 ± 0.2*	8.48 ± 0.6*
17 carbendazim	8.23 ± 0.2	13.62 ± 2.0	8.46 ± 0.3*	11.59 ± 0.1
170 carbendazim	10.54 ± 2.2*	16.78 ± 1.0*	14.61 ± 1.5*	8.47 ± 0.6*

Note: Within each sampling date, significant differences between fungicide treatments and control were indicated by *($P \leq 0.05$) and **($P \leq 0.1$).

Results and discussion

Soil microbial activity and biomass

Soil microbial activity and biomass from soil treated with all three different dosages of captan, chlorothalonil and carbendazim were analyzed during 65 days of incubation. Tables 1 and 2 show, respectively, the variations in microbial activity and biomass with incubation time progress. During whole incubation period, microbial activity and microbial biomass ranged from 7.1 to 18.4 μg fluorescein g^{-1} soil and 0.36 to 0.82 mg C g^{-1} soil, respectively. Captan had little or no effect on both microbial activity and biomass. However, chlorothalonil applied at higher dosages significantly increased microbial activity at the end of time incubation. In addition, clorothalonil at 250 mg g^{-1} soil decreased microbial biomass on day 11 and 30. Except on 65 DAFT, the fungicide carbendazim applied at 170 mg Kg^{-1} soil also increased microbial activity and decreased microbial biomass between 11 and 30 DAFT ($P \leq 0.05$). Microbial activity differences observed between untreated soil and soil under fungicide application was unclear and of poor consistency. However, in general, these differences were observed in soil treated with the highest fungicide dosages. A possible explanation for these results might be that fungicide applications affect the activity of certain groups of microorganisms, by altering the initial soil microbial balance. Thus, higher dosages of fungicide can reduce microbial activity by having toxic effects on soil microorganims. However, other groups of microorganims can use both fungicide and dead microbial biomass as a nutritional source to increase soil activity.[4] Although FDA has been commonly used as an index of overall microbial activity in soil, our results have shown a quite different response to the three fungicide applications. While higher dosages of both chlorothalonil and carbendazim significantly altered soil microbial activity, we can not find a significant effect from soil under captan applications. This relatively limited non-target effect of captan on soil microbial biomass and activity also was reported by other authors.[24] Burrows and Edwards[25] found that carbendazim had little impact on microbial biomass and activity. Yun et al.[26] observed that chlorothalonil significantly reduced soil bacteria and

Table 2. Microbial biomass (mg C g^{-1} soil) in soil treated with different dosages of captan, chlorothalonil, and carbendazim.[1]

	Days after fungicide treatment (DAFT)			
Treatment (mg Kg^{-1})	*3*	*11*	*30*	*65*
Control	0.49 ± 0.10	0.67 ± 0.20	0.64 ± 0.12	0.63 ± 0.07
3.5 captan	0.57 ± 0.08	0.82 ± 0.07	0.76 ± 0.08**	0.70 ± 0.07
35 captan	0.42 ± 0.08	0.74 ± 0.16	0.68 ± 0.08	0.68 ± 0.08
350 captan	0.40 ± 0.08	0.56 ± 0.05	0.63 ± 0.07	0.59 ± 0.04
2.5 chlorothalonil	0.52 ± 0.03	0.79 ± 0.01	0.76 ± 0.05	0.70 ± 0.09
25 chlorothalonil	0.42 ± 0.06	0.81 ± 0.01	0.75 ± 0.06	0.65 ± 0.02
250 chlorothalonil	0.56 ± 0.05	0.43 ± 0.03 *	0.42 ± 0.04 *	0.64 ± 0.03
1.7 carbendazim	0.49 ± 0.06	0.66 ± 0.06	0.72 ± 0.03	0.61 ± 0.05
17 carbendazim	0.43 ± 0.02	0.77 ± 0.13	0.73 ± 0.04	0.66 ± 0.01
170 carbendazim	0.53 ± 0.03	0.36 ± 0.04 *	0.48 ± 0.04 *	0.65 ± 0.04

Note: Within each sampling date, significant differences between fungicide treatments and control were indicated by *($P \leq 0.05$) and **($P \leq 0.1$).

actinomycetes populations. Partially according to this, our data had shown no effect of captan on microbial biomass. In addition, generally, a decrease of soil microbial biomass was observed at higher dosages of both chlorothalonil and carbendazim.

Soil fungal biomass

Soil ergosterol has been widely used as marker molecule for the presence of viable fungi. As a marker molecule for fungal biomass, ergosterol has several advances. It is easy to extract and measure using HPLC, and it is rapid turnover in fungal dead cells.[27] Montgomery et al.[20] developed a protocol to evaluate soil ergosterol content by using a microwave-assisted extraction method (MAE). However, recently, other authors suggest that ergosterol content and fungal biomass are not always closely correlated. Thus, PLFA 18:2ω6,9 has been suggested as a marker molecule for fungi, because it is very common in fungi, while generally absent in bacteria.[28] In our study, PLFA 18:2ω6,9c coeluted the isomer 18:2ω9,12c. In consequence, it was not possible to evaluate correlation between 18:2ω6,9 and soil ergosterol content. Application of all three fungicides significantly decreased soil ergosterol content up to day 11 compared with the control treatment (Table 3). Soil ergosterol content ranged from 6.4 to 7.8 μg g^{-1} soil, 5.3 to 7.5 μg g^{-1} soil, and 4.6 to 8.3 μg g^{-1} soil for soil treated with captan, chlorothalonil and carbendazim, respectively. Soil ergosterol content of the untreated soil (control) ranged from 7.4 to 8.8 μg g^{-1} soil. Except with carbendazim applied at 170 mg Kg^{-1} soil, on 3 DAFT, there was no effect of fungicide treatments on soil ergosterol content. In general, the lower values of ergosterol were founded at the end of incubation period (65 DAFT). Our data showed a significant decrease of soil ergosterol at 11, 30 and 65 DAFT from soil treated with all three fungicides. At 30 DAFT ergosterol content from all three fungicide dosages of each fungicide treatment was significantly lower than untreated soil. Similar findings were observed when undifferentiated sandstone soil was treated with fungicide captan at 100 mg g^{-1} soil.[29]

Microbial community structure

The analysis of PLFA profiles in our study indicates a differentiation of microbial communities in soil in which the three fungicides were applied compared to untreated soil. In addition, this change was more evident in soil treated at higher fungicide dosages than field rates applications. In all three fungicide treatments, 24 individual PLFAs were reported (Fig. 1). One way analysis of variance(ANOVAs) for several individual PLFAs showed a significant effect depending on fungicide treatment. PLFA profiles were dominated by fatty acid a15:0, 16:0, and 18:1ω9 which together accounted for more than 50% of total PLFAs. Soil treated with captan significantly decreased both fatty acids i17:0 and cy17:0. Generally, higher dosages of both captan and chlorothalonil increased the fatty acid 18:1ω7. The application of fungicide carbendazim at both 17 mg Kg^{-1} soil and 170 mg Kg^{-1} soil increased 18:1ω9c. In addition, chlorothalonil decreased all three cyclopropyl fatty acids to monoenoic precursors ratio, saturated to monounsaturated ratio, and Gram-positive to Gram-negative ratio. To our knowledge, there are three reports about the effect of fungicides on soil microbial community structure. Allison et al.[30] found that fungicide benomyl applied at field rate had little impact on arbuscular mycorrhizal fungal colonization, and on soil microbial community composition assessed as the relative abundance of different PLFAs. Similar results were reported by Demanou et al.[31] In contrast, 18S rRNA PCR-DGGE analyses of soil treated with azoxystrobin, tebuconazole, and chlorothalonil revealed that a number of bands were absent in certain fungicide treatments.[32] Our results indicate that the impact of captan, chlorothalonil and carbendazim on individual PLFAs was variable, increasing with higher dosages of fungicide. PLFAs 18:1ω7c and 18:1ω9c

Table 3. Ergosterol content (μg ergosterol/g soil) in soil treated with different dosages of captan, chlorothalonil, and carbendazim.[1]

	Days after fungicide treatment (DAFT)			
Treatment (mg Kg^{-1})	*3*	*11*	*30*	*65*
Control	7.99 ± 0.7	8.47 ± 0.1	8.80 ± 0.4	7.44 ± 0.1
3.5 captan	7.85 ± 0.6	7.06 ± 0.7*	7.40 ± 0.3*	7.17 ± 0.3
35 captan	7.61 ± 1.0	7.43 ± 0.1*	7.74 ± 0.6*	6.55 ± 0.1*
350 captan	7.08 ± 0.2	6.59 ± 0.6*	6.44 ± 0.3*	6.59 ± 0.3*
2.5 chlorothalonil	7.03 ± 0.5	7.30 ± 0.1*	7.45 ± 0.2*	6.06 ± 0.3*
25 chlorothalonil	7.52 ± 2.5	7.27 ± 0.2*	7.41 ± 0.18*	6.86 ± 0.5
250 chlorothalonil	7.25 ± 0.5	6.15 ± 0.1*	5.35 ± 0.7*	5.70 ± 0.3*
1.7 carbendazim	8.34 ± 0.4	8.09 ± 0.5	7.90 ± 0.2 +	7.83 ± 0.2
17 carbendazim	7.23 ± 0.7	6.25 ± 1.1*	7.10 ± 0.8*	6.55 ± 0.8*
170 carbendazim	6.22 ± 0.3*	5.72 ± 0.7*	5.81 ± 0.7*	4.65 ± 0.3*

Note: Within each sampling date, significant differences between fungicide treatments and control were indicated by *($P \leq 0.05$) and **($P \leq 0.1$).

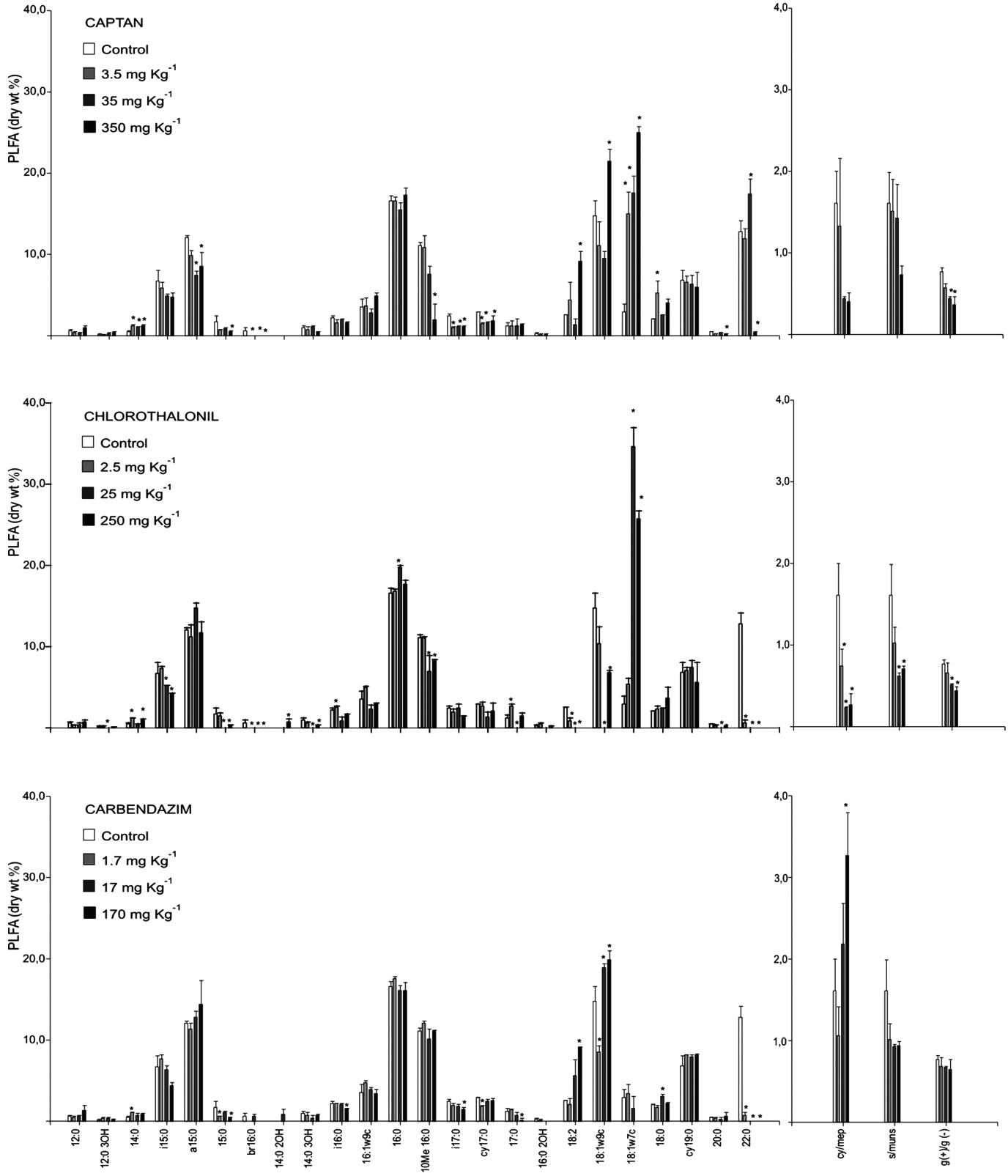

Fig. 1. Proportion of phospholipid fatty acid(PLFAs) in soil treated with different dosages of captan, chlorothalonil and carbendazim. Cy/mep = cyclopropyl fatty acids/monoenoic precursors; s/muns = total saturated/total monounsaturated fatty acids; g (+)/g(–)= Gram-positive/Gram-negative bacteria. Significant differences between fungicide treatments and control were indicated by asterisks ($P \leq 0.05$).

are considered as a Gram-negative indicator.[22] In our experiment, higher dosages of captan and chlorothalonil increased 18:1ω7c while higher dosages of carbendazim increased 18:1ω9c. However, Gram-positive to Gram-negative ratio only decreased in captan and chlorothalonil treatments. Additionally, captan and clorothalonil decreased PLFA 10Me 16:0, which is considered as an indicator of actinomycetes. In these respects, an increase of

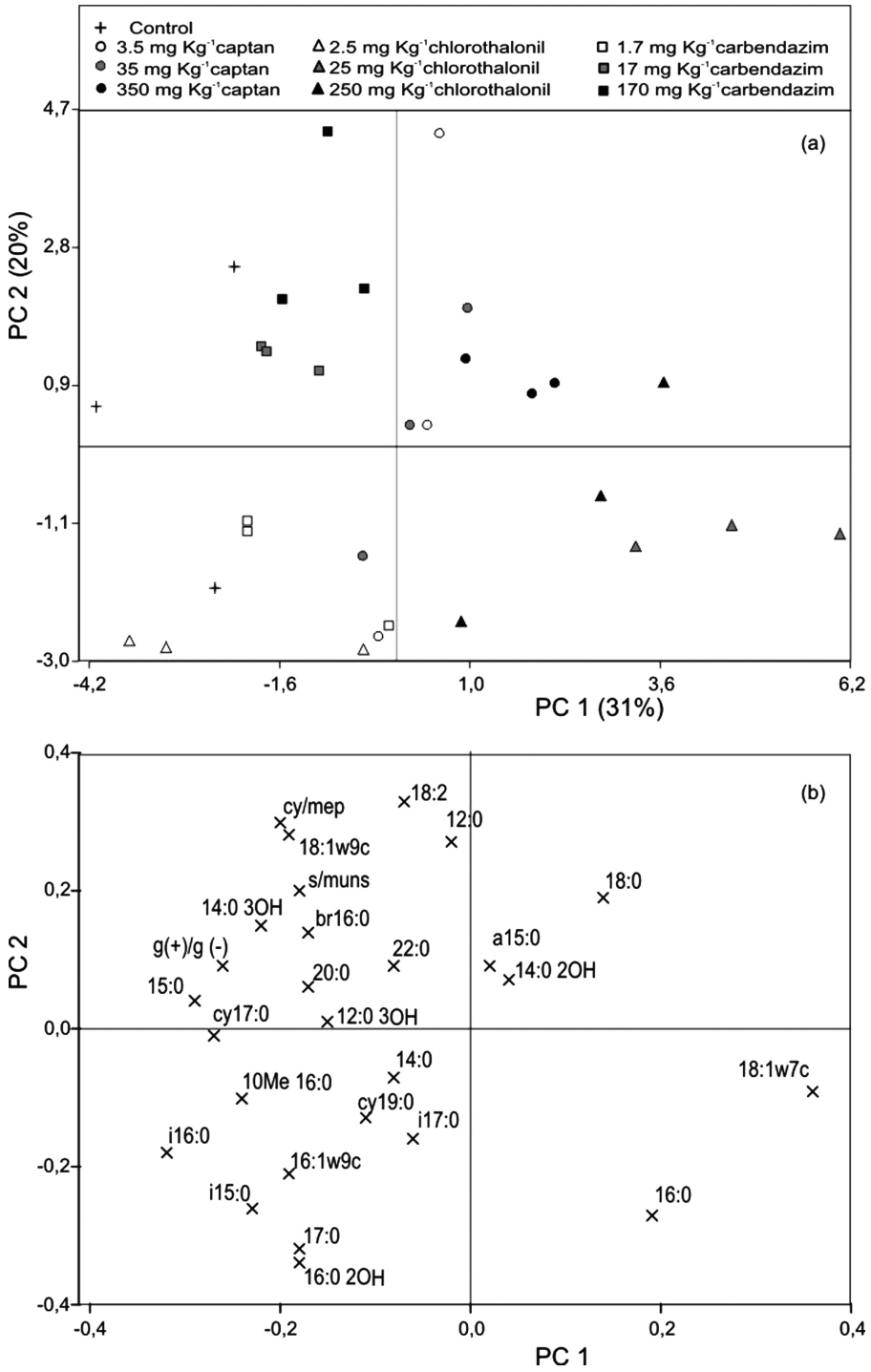

Fig. 2. (a) Principal component analyses (PCA) of all phospholipid fatty acids (PLFAs) detected in each sample of soil treated with different dosages of captan, chlorothalonil and carbendazim. Cy/mep= cyclopropyl fatty acids/monoenoic precursors; s/muns= total saturated/total monounsaturated fatty acids; g (+)/g(−) = Gram-positive/Gram-negative bacteria. (b) PCA showing loading values of individual PLFAs and ratios given in Figure 2(a).

monounsaturated fatty acids, lower dosages of branched-chain fatty acids and low level of methyl branching on the tenth C atom fatty acids also was reported in chemically perturbed soils.[33] Our results also showed that all three (captan, chlorothalonil and carbendazim) decreased a saturated straight-chain PLFA 22:0, an indicator of microeukaryotes. Recently, in support of this, Bending et al.[32] found that a small number of soil microeukaryotes (principally protozoa and fungi) were influenced by chlorothalonil application, as assessed by PCR-DGGE analysis. Microbial community composition was summarized for all three fungicide treatments by using PCA from proportion of signature PLFAs (Fig. 2a). PLFA composition varied with fungicide treatment, where the first principal component axis (PC1) explained 31% of the variance in data while the second principal component (PC2) explained 20%. Untreated soil and soil treated with carbendazim were positioned lower on PC1, while soils treated with captan were positioned higher. In contrast, on CP2, data showed no consistent differentiation between fungicide dosages. A major number of individual PLFAs were located at lower PC1 (Fig. 2b). Finally, our analysis on PCA showed that some saturated straight-chain fatty acid (15:0, 16:0, and 18:0), monounsaturated fatty acid (principally 18:1ω7c), and Gram-positive to Gram-negative ratio played a significant role in separation soil microbial communities according to fungicide treatments.

Conclusions

The application of all three captan, chlorothalonil and carbendazim fungicides altered soil microbial activity and fungal biomass. However, microbial biomass was only affected at higher dosages of both chlorothalonil and carbendazim. Analysis of PLFA profiles revealed that there was an alteration in the proportion of several individual PLFAs from soil treated with fungicide compared to untreated soil. Principally, this shift in soil microbial community structure was associated with both actinomycetes and Gram-positive bacteria populations. The finding that these fungicides can affect soil microbial community structure suggest that the sole use of selective techniques which measure a component of soil microbial such as microbial biomass or whole microbial activity, are likely to provide only a limited picture of the response of soil microbial communities to biocides applications. These data suggest that there is a need for a polyfaceted approach to further studies about responses of soil microbial community to environmental changes.

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