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Changes in Soil Chemical Properties and Microbial Activities in Response to the Fungicide Ridomil Gold Plus Copper

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Abstract: The purpose of the study was to investigate changes of soil chemical and biological properties changes resulting from a single application of the fungicide Ridomil Gold plus copper (Ridomil Gold plus)(mefenoxam 6% + copper oxide 60%) at the following rates 0.25, 0.5, 1, 2, and 10 g m^{-2}. Selected chemical properties generally differed between fungicide rates over longer incubation periods. Microbial activity indices (available N, ammonification rates and specific enzymatic systems) were more sensitive indicators of change. Values of these indicators generally increased with incubation period and decreased or increased at high rates. Significant changes in P availability occurred after 90 days of incubation at rates ≥ 1 g m^{-2}. Incorporation of the fungicide significantly increased NH_4+ levels in soil after 75 days of incubation. These changes stimulated soil microbial activity as evidenced by increased ammonification rates especially at long-term exposure. Of the enzyme activities studied, dehydrogenase and ß-glucosidase activities were the most sensitive to ridomil gold plus. This sensitivity was more pronounced with the dehydrogenase activity.

Key words: Ridomil gold plus, enzyme activities, soil chemical properties, field conditions, nitrogen transformation

Introduction

Ridomil gold copper plus (ridomil gold plus) is a new fungicide, which was recently put on the market by Syngenta Co, to replace the former ridomil plus 72 (copper oxide, 60% + metalaxyl, 12%) used to fight *Phytophtora* fungus in cocoa farms. It is a mixture of active ingredients copper oxide (60%) and metalaxyl-M (6%) (also called mefenoxam). Metalaxyl-M [methyl N-(2,6-dimethylphenyl)-N-(methoxyacethyl)-D-alaninate] is the R-enantiomer of metalaxyl and has been on the market since 1996 under various formulations and trade names including Ridomil gold, Fonganil gold, Apron XL, Subdue, and MAXX. It provides the same level of efficacy as metalaxyl but at half of the application rate. The introduction of metalaxyl-M may contribute to risk reduction for metalaxyl [1, 2]. Thus, metalaxyl-M is designed to replace technical metalaxyl in parts of the world where the registration of metalaxyl has not been renewed.

Ridomil gold plus is a new product and quantitative studies on its fate and effects are required. Numerous studies have documented microbiological changes in soil ecosystem as a result of pesticide application [3]. During a laboratory study comparing the effect of metalaxyl and mefenoxam on a soil quality sandy loam, it was found that both compounds exerted adverse effects on microbiological properties of the soil by decreasing the population of free nitrogen-fixing bacteria, by altering enzymatic activities and by stimulating the N

transformation and some plant nutrient availability in soil [4]. However, information is scarce concerning the effect of Ridomil gold plus on soil quality.

Since Ridomil gold plus is to replace ridomil plus 72, which was heavily used in Cameroon [5], it is important to consider its possible impact on soil quality and health, under more realistic conditions and with the formulation applied to the field. In assessing soil quality, chemical criteria have also been suggested, including potentially mineralizable N [6,8]. Enzyme activities have been considered to be sensitive indicators of soil quality [7, 9, 10]. The importance of soil enzymes resides in their relationship to soil microbiology, ease of measurement and rapid response to changes in soil management. It is conceptually wrong to rate a single enzyme activity as criteria of soil quality or soil microbial activity [10].

Thus, many enzyme activities should be considered. Phosphatase activities are considered especially useful indicators of both positive and negative effects of soil management practices on soil quality [9,11]. Glucosidase activity is often included in studies searching for sensitive biomarkers of soil quality [12]. Its activity is considered to be a useful index for measuring side effects of pesticides on microbial activity in soil [13]. Dehydrogenase activity in soil provides an index of the overall microbial activity [14].

In this study both chemical and microbiological soil properties were used for the evaluation of soil quality changes under field conditions as a result of single application of various doses of ridomil gold plus to an agricultural soil. The specific objective of this study was the determination of the effects of ridomil gold plus amendments on selected soil quality parameters including N and P transformation processes, and soil enzyme activities.

Materials and Methods

Experimental Site

The study was conducted in Yaounde-Cameroon on an agricultural plot (Table 1), which had not received any pesticide application for at least ten years. This site has been under continuous cultivation since 1993. The crops grown irrespective of season included maize, peanut, sweet potato, cassava, and beans. The study site was divided into six blocks. Each block contained replicates of all fungicide treatments. Plots in the blocks were organized according to the "Latin Square" model of randomized blocks [15].

This arrangement of plots presented a complete randomization, as treatments as well as controls were distributed at random in blocks and in columns. In order to evaluate undesired influences such as soil differences amongst blocks and border effects, each treatment occurred once in each soil block. Each block and each column contained all the treatments. Each plot measured 1 m x 1.5 m. The experiments began at the onset of the rainy season and ended at the beginning of the following dry season.

Table 1: General characteristics of soil.

Texture analysis (USDA)	*Clay*
Clay (< 2 µm) (%)	49.8
Silt (< 50-2 µm) (%)	13.6
Sand (2000-50 µm) (%)	36.6
pH_{H2O} (1:2.5)	5.79
pH_{CaCl2} (1:2.5)	4.72
Maximum water holding capacity (MWHC) (%)	41.18
Bulk density (g/m³)	1.27
Field capacity (cm³/cm³)	0.39
Organic C (% dry soil)	1.43
C/N	15.8
Cation exchange capacity (meq/100g dry weight)	4.02
Moisture (%)	17.23

Application of the Fungicide in the Field

Ridomil gold plus (6% mefenoxam and 60% copper oxide, both active ingredients) in wettable powder formulation was obtained from a local vendor in Yaounde, Cameroon. All other chemicals used in the study were analytical grade from Merck Co. or Aldrich Chemical Co.

Calculated amounts of Ridomil gold plus corresponding to rates of 0.25, 0.5, 1, 2 and 10g/m² dry soil were weighed and mixed thoroughly and separately in 1500 ml of distilled water in a watering can and uniformly applied once by hand spray to the prepared surface of plots, as recommended by the manufacturer. These doses of fungicide were selected to cover the range of ridomil gold plus recommended field dosages for various crops [16].

Soil Sampling and Preparation

Three soil cores were taken at random from each plot at each incubation period (7, 14, 30, 45, 75 and 90 days) from the 0 to 10 cm depth using a plastic soil auger. These samples were bulked and homogeneously mixed in a plastic bucket. The soil was gently air-dried to the point of soil moisture suitable for sieving. After sieving to a maximum particle size of < 2 mm, the soil was temporary conserved in sealed plastic bags. Exchangeable NH_4^+ was determined immediately after

sampling on field moist and sieved sub-samples. Sub-samples for the determination of available P ground and sieved to pass a 0.5 mm sieve. The soils for enzyme activity analysis were kept field moist and stored frozen until analysis. The determination of gravimetric soil moisture content was performed concurrently with each parameter analysis.

Chemical Analyses

Ammonium-N was extracted by 2M KCl following the procedure already described [17], and quantified using a colorimetric procedure [18]. Soil samples for available P were also extracted with $NaHCO_3$, pH 8.5 [19], and analyzed for available P spectrophotometrically at 882 nm. Results of $NH4^+$-N and available P are reported on an oven dry-weight basis, determined by drying the soils for 24h at 105°C.

Progress curves [P versus t, where P is the reaction product, (NH_4^+-N or P), and t is the incubation time], were plotted and used for calculating the mineralization rates processes. Since the progress curves were in general biphasic, the exponential phases of the plots were considered for rates determinations. Thus, the first portion (8-12 days) of the P-mineralization plots was used to derive the rate of P-mineralization. The second portion (30- 90 days) of the N-mineralization plots was used to determine the rate of ammonification under ridomil gold plus stress.

Enzyme Activities

Acid and alkaline phosphatase activities were determined according to reported spectrophotometrical methods [20,21] with slight modifications. Soil samples (1 g) were mixed with 4ml of modified universal buffer (MUB) of pH 6.5 and pH 11 for acid and alkaline phosphatase assays respectively, and 0.05M p-nitrophenyl phosphate (1 ml) and incubated for 1 h at 37± 1°C. Then, 0.5M $CaCl_2$ (1ml) and 0.5 M NaOH (4ml) were added and the mixture was centrifuged at about 1500 x g for 10 min. The p-nitrophenol (PNP) in the supernatant was determined colorimetrically at 400 nm. Toluene was not included in the procedure because it has been shown to increase both acid and alkaline phosphatase activities [22].

ß-Glucosidase activity was measured following a colorimetric method [23]. Four ml of MUB (pH 6.0) and p-nitrophenyl-ß-D-glucopyranoside (1ml) were added to soil (1g) and the reaction mixture was incubated without toluene at 37± 1°C for 1h. Thereafter, the method was the same as described above for acid and alkaline phosphatase activity.

Dehydrogenase activity in soil was determined following an already described spectrophotometric method [24] by reduction of 2,3,5-triphenyltrazolium (TTC). Each soil sample (20 g) was thoroughly mixed with $CaCO_3$ (0.2 g) to ease methanol extraction and 6 g of this mixture were treated in triplicate with 3% (w/v) 2,3,5-triphenyltrazolium chloride (1 ml) and incubated for 24 h at 37 ± 1°C.

The triphenyl formazan (TPF) formed was extracted quantitatively from the reaction mixture with methanol and assayed at 485 nm in a Hach DR 2000 spectrophotometer. Since copper may interfere with dehydrogenase assay [25], the TPF quantities produced during the reaction with copper contaminated samples were corrected by compensating for amount of TPF complexated by Cu. This was achieved by incubating pure TPF (1000 µg) with 5 g of Cu-non contaminated (control) and Cu-contaminated soils. This mixture was subsequently analyzed as described above. The amount of TPF complexated by Cu was calculated as the difference between control soil TPF and Cu-contaminated soil TPF. This amount was then added to those obtained from the analyses of field samples. Results of all enzyme activities are reported on an oven dry-weight basis, determined by drying the soils for 24 h at 105°C.

Statistical Analysis

The results at each sampling period were compared using analysis of variance (ANOVA). When treatment responses differed significantly from controls ($P<0.05$), multiple comparisons were made using paired-t test procedure [26]. Determination of effect concentrations for significant responses was based on the nominal test chemical concentration at each sampling period.

The no-observable-effect concentration for each significant response (NOEC, the highest tested concentration where the response was not significantly different from controls) and the lowest-observable-effect concentration (LOEC, the lowest concentration where the response was significantly different from controls [27] were determined for enzyme experiments. The IC_{50} (Median inhibitory concentration at which a 50 % reduction in response relative to controls is predicted) values were determined for microbial responses adversely affected by ridomil gold plus using least squares linear regression of percent of control of microbial responses against the logarithm of test chemical concentrations.

Results and Discussion

Effect on N and P Mineralization Processes

Figures 1 and 2 show the variations in NH_4^+-N and available-P concentrations with respective incubation time (progress curve). The nitrogen mineralization (ammonification) illustrated in Figure 1 showed kinetics characterized by fast rate from 30 to 45 days of incubation. This phase of rapid formation of NH_4^+-N in soils was

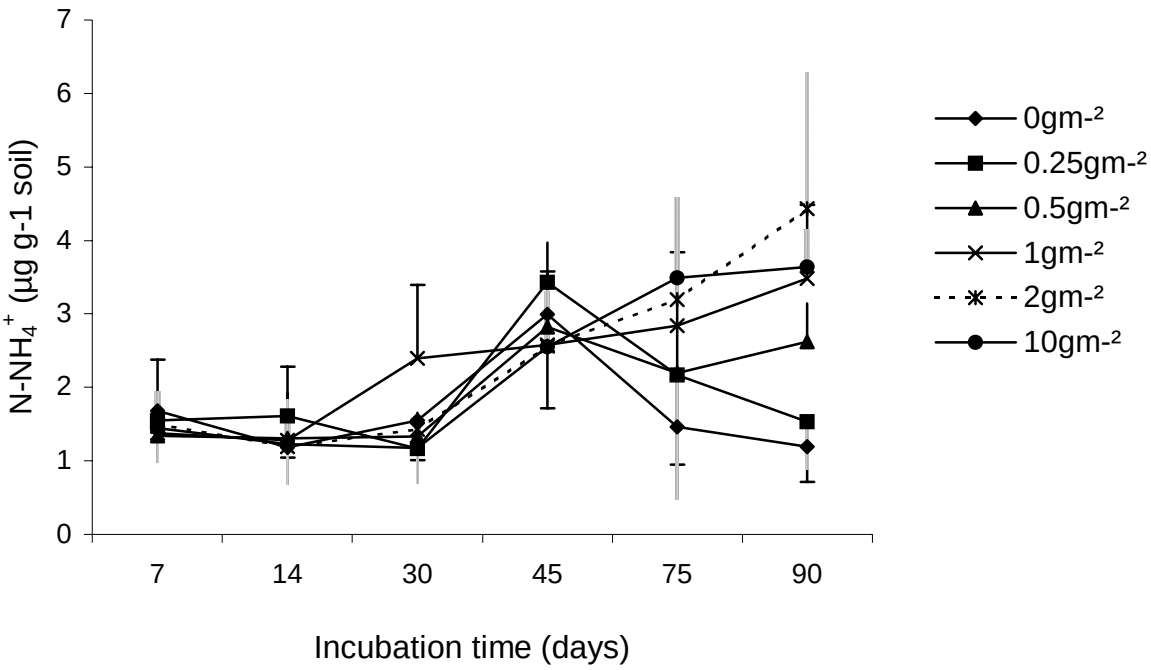

Fig. 1: Changes in NH_4+-N levels in the samples during incubation at different concentrations of Ridomil gold plus.

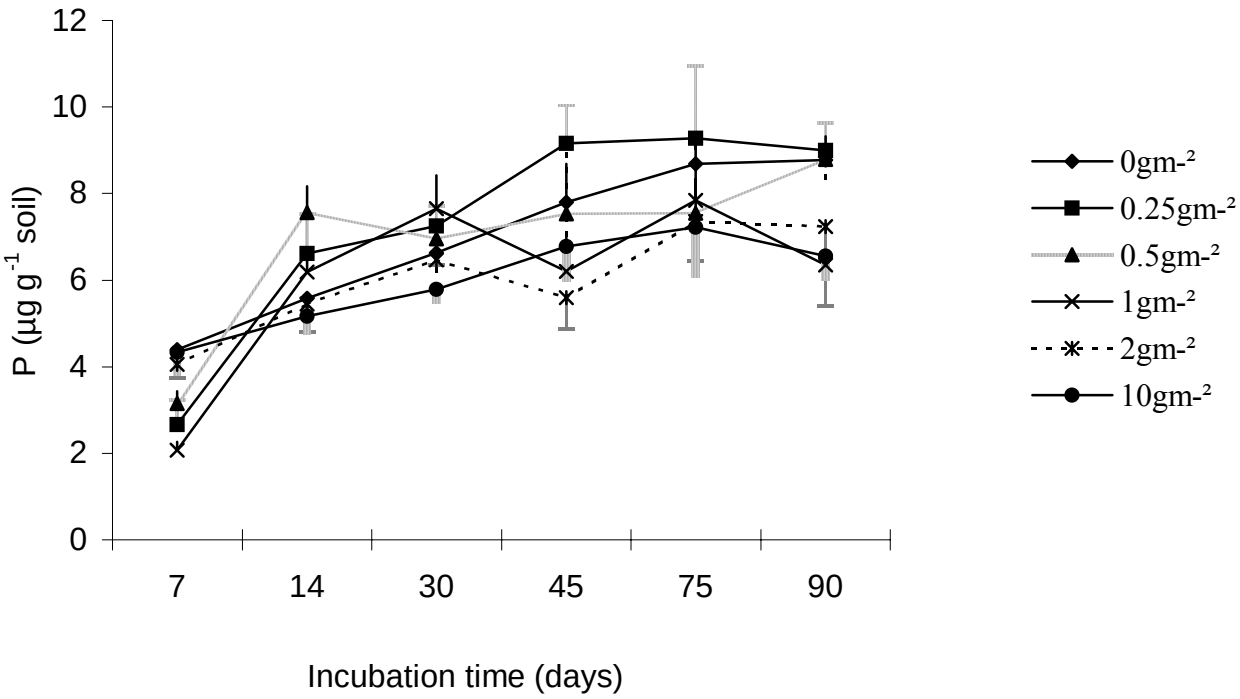

Fig. 2: Changes in available P levels in the samples during incubation at different concentrations of Ridomil gold plus.

followed by a somewhat less rapid formation phase above 45 days of incubation for ridomil gold plus treatment, and above 0.25 g/m² dry soil. A rapid depletion in the NH_4^+-N concentration was observed in the control and 0.25 g/m² dry soil treatment plots after 45 days of incubation.

The organic P mineralization shown in Figure 2 reveals kinetics characterized by steady increases in available P levels, for all soil treatments after 14 days of incubation. The greatest accumulation of P after 30 days of incubation was observed in soils treated with ridomil gold plus at the rate 0.25 g/m². A slight decrease in available P levels was observed with doses of the fungicide ≥ 0.5 g/m² after 14 days of exposure. A significant decrease in these levels was only observed after 90-day exposure with doses greater than 1g/m².

Ridomil gold plus stimulated the rate of ammonification at concentrations above 0.25 g/m² dry soil, 30 days after the start of the experiment. Similar observations were also made by other workers with insecticides [28, 29]. It has been reported that mefenoxam increases ammonium-N levels in sandy loam and sandy clay soils after 14 days of exposure as a result of stimulation of the growth of ammonifiying bacteria [30]. This situation may probably occur through killing of a part of microflora and increasing of NH_4^+-N by surviving part of the microflora. In fact, soil microbial community is a complex picture of interwoven relationship between micro-organisms of different trophic levels. Some microbial species are able to use an applied pesticide as a source of energy and nutrients to multiply, whereas the pesticide may well be toxic to other organisms [6].

Inhibition of nitrate could be another way of NH_4^+-N accumulation in soil. In fact, copper interferes with N metabolism in soils or in plants. Exposures of plants (Vitio vinifera) to low concentration of copper (5 µg/l) produces dramatic change in N metabolism with a reduction on nitrate and free amino acid contents in roots and leaves which reflects the reduction of nitrate reductase to a negligible activity [31]. It was reported that high concentrations of metalaxyl inhibited the nitrification in soil [32]. Moreover, high levels of mefenoxam and metalaxyl (1000 µg/g of soil) were shown to severely inhibit nitrification [4]. Ridomil gold plus also stimulated the rate of organic P mineralization at concentration < 2 g/m² dry soil and slightly inhibited it at a very high concentration (10 g/m² dry soil). An increase in organic P mineralization in soil was reported following the application of organic insecticides viz, phorate, carbofuran and fenvelerate [33].

Effects on Soil Enzyme Activities

Generally, the application of ridomil gold plus did not significantly affect both acid and alkaline phosphatase activities (Table 2), except on the 7th day of the incubation when a stimulatory effect on acid phosphatase activity was observed for a concentration of 0.5 g/m² dry soil, and on the 90th day of incubation when alkaline phosphatase activity was significantly inhibited at a concentration 10 g/m² dry soil. When assessing in the laboratory, the effect of mefenoxam on soil enzymes, it was reported the stimulation of acid phosphatase activity and the inhibition of alkaline phosphatase activity at incubation periods of 14 to 90 days of exposure at higher doses of mefenoxam (>100 µg/g dry soil) [30].

Acid and alkaline phosphatases are exo-enzymes and may be protected from degradation by adsorption to clays or to humic substances [34]. This protection of these exo-enzymes in addition to the continuous production by plant roots may result in their insensitivity towards the fungicide application. Some authors have suggested that it is even difficult to study the effects of pesticides on extracellular enzyme activities in soil [10]. Alkaline phosphatase is mostly found in micro-organisms and animals [22, 35]. The slight decrease observed in this enzyme activity at high doses after 14 days of incubation could be ascribed to the suppression of a sensitive fraction of the soil biota. Ridomil gold plus contains copper as one of its active ingredients. Trace metals affect the activity of extracellular enzymes by modifying the conformation of the proteins [36]. Metal ions also can inhibit enzyme reactions by complexing the substrate [37]. At high levels, both essential and non-essential metals can damage cell membranes, alter enzyme specificity and disrupt cellular functions [38].

Generally, ß-glucosidase activity was significantly affected by ridomil gold plus at concentration ≥ 0.5 g/m² dry soil after 45 days of incubation (Table 3). However, ß-glucosidase was less sensitive than dehydrogenase and this sensitivity in general, followed a dose-response pattern. Seven days following the application of ridomil gold plus, dehydrogenase activity in treated samples with concentrations ≥ 0.5 g/m² dry soil was significantly ($P \leq 0.05$) less than that of the untreated samples (control), indicating a severe inhibitory effect of this fungicide on the dehydrogenase activity (Table 3). This inhibition remained irreversible at all sampling periods and followed a dose-response pattern, with higher doses showing more severe inhibitory effect on this enzyme activity. This is in agreement with many reports on the adverse effects of pesticides including fungicides on the dehydrogenase activity [30, 39, and 40].

The IC_{50}, NOEC and LOEC values for all significant enzyme activities responses are shown in Table 4. Effect levels were determined based on the nominal average fungicide concentration. The lowest IC_{50} value for ß-glucosidase recorded on the 75th day of incubation was 5.42 g/m². It was reported that β-glucosidase activity was sensitive to metals (Zn and Cu) derived from the addition of sewage sludge in low organic matter soil [41]. This inhibitory effect could be caused by the suppression of ß-

Table 2: Effects of ridomil gold plus on soil acid phosphatase and alkaline phosphatase activities.

Ridomil gold plus ($g\ m^{-2}$ soil)	% Acid phosphatase activity[a] (days after application)						% Alkaline phosphatase activity[b] (days after application)					
	7	14	30	45	75	90	7	14	30	45	75	90
0	100	100	100	100	100	100	100	100	100	100	100	100
0.25	117	105	105	99	99	101	102	101	101	106	104	100
0.5	130*	100	103	101	102	99	100	100	98	109	115	117
1	99	99	101	100	101	103	84	101	111	102	95	99
2	100	100	100	99	100	102	99	98	94	99	94	85
10	90	98	92	90	93	93	104	74	86	89	85	62*

Data expressed as percentages in relation to their respective control (no fungicide addition).

*Parameter with fungicide addition significantly different ($P \leq 0.05$) from no fungicide addition parameter, using the paired Student t-test.
[a] Acid phosphatase activity in control samples after 7, 14, 30, 45, 75 and 90 days corresponded to 44.2, 343.1, 363.2, 422.5, 306.2 and 318.8 µg pNP g^{-1} soil respectively after incubation;
[b] Alkaline phosphatase activity in control samples after 7, 14, 30, 45, 75 and 90 days corresponded to 157.3, 155.3, 165.4, 123.3, 112.0 and 166.6 µg pNP g^{-1} soil respectively after incubation.

Table 3: Effects of ridomil gold plus on soil dehydrogenase and ß-glucosidase activities.

Ridomil gold plus ($g\ m^{-2}$ soil)	% Dehydrogenase activity[c] (days after application)						% ß-glucosidase activity[d] (days after application)					
	7	14	30	45	75	90	7	14	30	45	75	90
0	100	100	100	100	100	100	100	100	100	100	100	100
0.25	85	81	92	97	88	94	91	78	97	95	92	87
0.5	64*	80*	94	99	76*	71*	91	94	83	98	82*	72*
1	57*	84	99	98	64*	73*	98	106	81	66*	63*	65*
2	52*	62*	66*	65*	53*	69*	96	93	87	70*	54*	68*
10	38*	49*	34*	38*	42*	48*	97	96	69*	53*	49*	64*

Data expressed as percentages in relation to their respective control (no fungicide addition).

*Parameter with fungicide addition significantly different ($P \leq 0.05$) from no fungicide addition parameter, using the paired Student t-test.
[c] Dehydrogenase activity in control samples after 7, 14, 30, 45, 75 and 90 days corresponded to 400, 478.2, 472.3, 465.7, 595.2, and 505. µg 1TPF g^{-1} soil respectively after incubation;
[d] ß-glucosidase activity in control samples after 7, 14, 30, 45, 75 and 90 days corresponded to 46.8, 34.6, 38.1, 31.6, 41.3 and 44.0 µg pNP g^{-1} soil respectively after incubation.

Table 4: IC_{50}, NOEC, LOEC (g m^{-2} soil) for soil enzyme activities to fungicide stress at acute and chronic exposures.

	Fungicide toxicity (g m^{-2} soil)	Exposure period (days)					
		7	14	30	45	75	90
DHA	IC_{50}	2.8	10.3	5.7	10.8	4.3	7.9
	NOEC	0.3	0.3	1.0	0.0	0.3	0.3
	LOEC	0.5	0.5	2.0	0.3	0.5	0.5
ß-glu	IC_{50}	ND	ND	78.2	8.6	5.4	89.4
	NOEC	ND	ND	2.0	0.5	0.3	0.3
	LOEC	ND	ND	10.0	1.0	0.5	0.5
Acid Phos.	IC_{50}	ND	>10 000	ND	249.8	542.6	253.4
	NOEC	ND	ND	ND	ND	ND	ND
	LOEC	ND	ND	ND	ND	ND	ND
Alk Phos.	IC_{50}	>10 000	19.50	60.7	48.8	>10 000	13.7
	NOEC	ND	ND	ND	ND	ND	ND
	LOEC	ND	ND	ND	ND	ND	ND

(DHA: dehydrogenase activity; ß-glu: ß-glucosidase activity; acid phos: acid phosphatase activity; Alk. Phos.: alkaline phosphatase activity).
IC_{50} = Median inhibitory concentration at which a 50 % reduction in response relative to controls is predicted.
NOEC = No observed effect concentration = The highest toxicant concentration at which the response is not significantly different from control.
LOEC = Lowest observed effect concentration = the lowest toxicant concentration at which the response differs significantly from control.
ND = No determination.

glucosidase producing microbes or direct inhibition of the enzyme. It was also reported that composition of microbial communities (fungi, eubacteria and actinomycetes) was still affected after 50 years of Cu contamination soils [42].

Dehydrogenase activity was more sensitive to ridomil gold plus throughout the experiment. This sensitivity was more pronounced at shorter incubation periods (7 and 14 days), indicating a strong inhibitive effect with IC_{50} values of 2.8 and 10.2 µg/g respectively. Mefenoxam was found to significantly inhibit dehydrogenase activity at high doses [30]. Cu was also found to inhibit dehydrogenase activity with IC_{50} values of 24.77 mg Cu/ kg of soil [43]. Cu concentrations varying from 5-25 mg/L strongly inhibited the fungal mycelial growth and activity of enzymes like glucose-6 phosphate dehydrogenase and malate dehydrogenase [44].

The inhibitory effect of a pesticide on an enzyme activity can last as long as the pesticide concentration is sufficiently high to permit its interaction with the enzyme molecule. However, the aerobic soil metabolism half-life of metalaxyl was determined to be about 40 days, acid metabolite accounting for as much as 53.6% of the applied material at 66 day and there after degraded to 23% at 360 days [45]. Moreover, Cu is known to be persistent in the environment. The application of the fungicide could result in the suppression of part of soil living biota. Dehydronenase occurs intracellularly in all living microbial cells and it is linked with microbial respiratory processes [46] and thus, this enzyme activity can reflect the physiologically active bacteria, fungi and actinomycetes [47].

Conclusions

Under field conditions, ridomil gold plus, applied at commercially recommended rates, exerted an adverse effect on microbiological properties of soil as manifested by the observed altered enzymatic activities. This fungicide stimulated N and organic P mineralization

whereas dehydrogenase and ß-glucosidase activities were negatively affected. Because of their sensitivity, they can serve as a useful early warning bioindicator for the side effects of ridomil gold plus on soil microbiological activity, as previously suggested for mefenoxam and metalaxyl [30].

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