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RESEARCH ARTICLE

Copper availability and selective microbiological properties of an intensively cultivated ultisol in Nuwara Eliya

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Abstract: A study was undertaken to assess the effect of copper on microbiological properties of intensively cultivated vegetable fields at Nuwara Eliya. Soil samples were collected from six cultivated fields and an undisturbed forest were assessed for pH, total and DTPA extractable Cu, biomass nitrogen (BN) and substrate induced respiration (SIR). Total and Cu-resistant bacteria were enumerated using four agar media. Soil pH of experimental soils ranged from 4.44 to 5.44. Organic C content in cultivated soils varied from 1.8 to 3.3 % and it was 6.8% in forest soil. Total and DTPA extractable Cu contents varied from 14.4 to 25.6 mg kg^{-1} and 1.2 to 4.5 mg kg^{-1}, respectively. Forest soil showed the highest SIR (46 µg CO_2 g^{-1} soil h^{-1}) and BN (267 µg N g^{-1} soil). The highest Cu-resistant bacterial population was 0.43% of the total population reported for the forest. Building up of Cu and increasing of population of Cu-resistant bacteria was not evident due to cultivation. The percentage of Cu-resistant bacteria correlated positively with DTPA extractable Cu (R = 0.49) suggesting that threshold Cu levels for bacterial growth in experimental soils remain within the range of extractable Cu concentrations reported. This relationship was influenced by soil organic C content and pH. Tryptic soy agar (TSA) medium produced higher percentages of Cu-resistant bacteria for both forest and cultivated soils. Those percentages showed linear relationships with total Cu (r^2 = 0.95) and percentage of DTPA extractable Cu (r^2 = 0.94) indicating suitability of the TSA medium to enumerate Cu-resistant bacteria.

Keywords: Bacteria, copper, cultivated ecosystems, organic carbon, pH.

INTRODUCTION

Accumulation of heavy metals in soil is a common external disturbance which affects soil quality. Copper is known to accumulate in soils of intensively cultivated agricultural systems applied with high doses of fungicide and poultry manure[1,2]. Copper exists in the soil environment in different forms; exchangeable, sorbed, organically bound, precipitated, and residual[3-7]. The fraction of Cu in soil solution is governed directly by the quality and the quantity of organic matter, clay type, amount of Fe and Al oxides in soil and indirectly by soil pH[3, 5, 8, 9]. Copper speciation thus varied among soils with different soil characteristics[6, 10].

Metals in the soil environment are beneficial for microbial growth but detrimental when their concentrations reach toxic levels. In general, metal stress in agricultural soils is known to reduce the size and the activity of microbial biomass and alter the composition of microbial community structures[11,12]. A range of microbiological properties had been suggested as indicators of metal stress conditions in the soil environment. Examples are substrate induced respiration,[12] nitrification,[13] microbial biomass[14] and carbon utilization efficiency[15]. However, micro-organisms may detoxify harmful metals through various biochemical mechanisms and develop resistance against metals to a certain degree[12]. This has been demonstrated for soils contaminated with metals naturally and experimentally[14,16,17]. Therefore, populations of heavy metal resistant bacteria and fungi have been proposed as indicators of metal pollution in natural and disturbed soil[12, 16, 18].

Vegetable cropping systems in Nuwara Eliya receive a large quantity of agro-chemicals throughout the growing season. Soil in this area has been classified as ultisol, which is a predominant soil order in humid tropics. These soils are characterized with low pH and high 1:1 type clay minerals that influence metal speciation to a greater extent. As a result, metal stressful conditions may exist in soils under vegetable cropping systems. Therefore, a study

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was undertaken to assess the variability of Cu fractions and microbiological properties of intensively cultivated ultisol and the possibility of developing resistance to Cu in bacteria. For comparison, an undisturbed forest soil, which did not receive agrochemicals, was included.

METHODS AND MATERIALS

Site description and soil sampling: The studied site located in Nuwara Eliya, is 1900 m above mean sea level. This area is characterized with hilly landscape and has a mean annual temperature of 16°C and mean annual rainfall of 2500 mm. The soil is classified as Typic paleudults. Soil in this area is strongly acidic in reaction and kaolinite has been reported as the dominant clay mineral associated with 10% or more gibbsite[19, 20]. Potato (*Solanum tuberosum*) is the main crop grown in the studied area, at least twice a year with high inputs of chemical fertilizers, cattle and poultry manures and fungicides. Soil samples were collected from a 0-10 cm depth of six fields at the harvesting stage of potato. The selected fields have been cultivated for different durations with varying management practices (Table 1). Soil samples were also collected from a virgin montane forest in the upperslope position of the same landscape. Six composite soil samples were collected from each cultivated field and forest (total of 42 samples) and stored at 4°C until analyzes commenced.

Soil chemical properties: Air dried and sieved soil samples were used to assess pH, carbon and Cu fractions. Soil pH was measured by glass electrode[21] in a suspension of 2M KCl solution (1:2.5) and total organic C content by wet digestion[22]. Total Cu in soil was extracted by digesting 2 g of air dried soil in 20 mL of 4N HNO_3 solution for four hours at 80°C[23]. Potentially bioavailable Cu fraction was extracted using DTPA –TEA solution (0.005 M diethylenetriaminepentaacecetic acid, 0.1M triethanolamine, and 0.01 M $CaCl_2$ at pH 7)[24]. Both Cu fractions were analyzed by Atomic Absorption Spectrophotometry (Shimadzu, AA6200) with a minimum detection level of 0.01 ppm.

Soil microbiological properties: Substrate induced respiration (SIR) was determined by adding glucose to fresh soil at a rate of 200 µmol glucose g^{-1} soil[25]. Microbial biomass nitrogen (BN) was determined using chloroform fumigation extraction method[26]. The extracted nitrogen forms were reduced to NH_4^+-N and analyzed using the colorimetric method[27]. The populations of culturable bacteria were enumerated by plating aliquots of serial dilutions to tryptic soy agar (TSA) medium amended with 100 mg of cycloheximide L^{-1}. The same medium was amended with $CuSO_4$ to achieve a final concentration of 5 mM and used to enumerate Cu-resistant bacteria. Plates were incubated for 3-7 d at 28°C and single colonies were counted. An attempt was also made to assess the suitability of different agar media to estimate the Cu-resistant bacterial population. For this purpose, three additional agar media, tryptic soy broth (TSB), potato dextrose yeast-extract agar (PDYA) and peptone-glucose agar (PGA) amended with $CuSO_4$ and cycloheximide were used. These agar plates were inoculated with soil extractants of the forest and the cultivated field 'F'.

Statistical analysis: Means were compared with the Duncan mean separation test at 5% probability level for all the measured properties using SPSS (Version 13, 1998) analytical software. Means and the least significant differences at the 5% level were calculated by one – way ANOVA for four different bacterial media in forest and field 'F'. Pearson correlation coefficients were computed for selective variables.

Table 1: The number of years of cultivation, landscape position and intensity of cultivation of selected fields

Field	Years of cultivation	Landscape position	Intensity of cultivation[a]
A	>45	shoulder	high
B	30	shoulder	high
C	30	footslope	high
D	10	shoulder	low
E	10	footslope	high
F	-	shoulder	high

[a] High intensive cultivation, refers to fields with year-round continuous cultivation, received agrochemicals frequently at a rate of exceeding the recommended levels of the Department of Agriculture, whereas low intensity refers to fields that fallowed for 1 – 2 months per year and received agrochemicals at or less than the recommended dosages.

Table 2: Soil chemical properties of cultivated and forest soils

Field	Soil pH	Organic C	Cu contents		
			Total	DTPA extractable	DTPA extractable / Total Cu
		(%)	(mg kg^{-1} soil)		(%)
A	4.89^{abc}	1.8^{c}	25.6^{a}	2.2^{bc}	8.6^{b}
B	5.44^{a}	2.7^{bc}	14.4^{b}	2.0^{bc}	13.9^{b}
C	5.29^{ab}	3.3^{b}	15.3^{b}	1.2^{c}	7.8^{b}
D	4.63^{c}	3.3^{b}	16.1^{b}	1.8^{bc}	11.2^{b}
E	4.78^{bc}	3.2^{b}	17.3^{ab}	1.9^{bc}	11.0^{b}
F	4.44^{c}	1.8^{c}	18.3^{ab}	4.5^{a}	24.6^{a}
forest	5.41^{ab}	6.8^{a}	19.3^{ab}	3.2^{ab}	16.6^{ab}

Means (n=6) given in a column followed by different letters are significantly different (subset for α= 0.05)

RESULTS AND DISCUSSION

Soil chemical properties

In this study, soil from a forest located in the same landscape was chosen as the reference site. Although a forest is not the ideal reference site as the vegetation may also affect the metal status in the soil, the forest was chosen as it was not influenced by agronomic practices imposed on the cultivated fields.

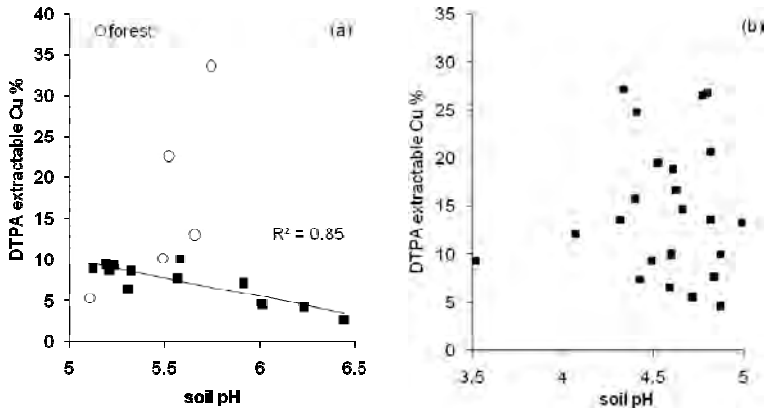
All the experimental soils in the seven fields had acidic soil reaction and their mean pH values ranged from 4.44 to 5.44 (Table 2). The mean total organic C content in the cultivated soils varied from 1.8 to 3.3% and they were significantly lower than the forest soil (6.8%) (Table 2). Fields 'A' and 'F' showed significantly lower C contents than fields 'C', 'D' and 'E'. The total and DTPA extractable Cu concentrations in forest soil were 19.3 and 3.2 mg kg^{-1}, respectively. The total Cu contents of cultivated fields were comparable to that of the forest soil (Table 2). Therefore, building up of Cu was not evident despite continuous cultivation with metal inputs. One possible reason would be crop uptake over the growing season. The DTPA extractable Cu contents of the cultivated fields varied from 1.2 to 4.5 mg kg^{-1} (Table 2). The minimum DTPA extractable and total Cu contents reported for the manured fields of Belgium, which are considered as contaminated, were 3.2 and 17 mg kg^{-1} soil, respectively[16]. The highest DTPA extractable Cu content observed for field 'F' (4.5 mg kg^{-1}) could be attributed to its very high acidic pH and very low organic C content. The percentage of DTPA extractable Cu was highest in the field 'F' (24.6%) and followed by the forest soil (16.6 %) (Table 2). In addition to high total Cu, low pH and low organic C content of field 'F' may have resulted in a higher fraction of DTPA extractable Cu.

A linear relationship was established between pH and percentage of DTPA extractable Cu for soil samples having a pH greater than 5 (Figure 1a), but forest soils with high organic matter content remained as outliers. In cultivated soils, high organic matter content at high pH condition reduce Cu^{2+} sorption[4] and free metal activity[14]. However, organic C content did not correlate significantly with DTPA extractable Cu fraction of the experimental cultivated soils indicating that it was governed primarily by soil pH.

Soil microbiological properties

Microbial biomass in soils is generally governed by the labile C pool[28]. Forest soil showed the highest BN content (267 µg N g^{-1} soil) (Table 3) which is not unrealistic in comparison to BN content of 120 µg g^{-1} soil, recorded for a tropical forest soil with an organic C content of 2%[29]. Ocio and Brookes[30] reported that BN values of continuously cultivated soils, which have organic C contents in the range of 1.3 to 2.8%, varied from 20 to 80 µg N g^{-1} soil. In this study, fields 'A' and 'F' with 1.8% organic C content showed BN content of 173 and 58 µg N g^{-1} soil, respectively. The former may be either due to high nitrogen availability or due to an over-estimation. In addition to significantly low organic C content and acidic pH, higher Cu availability may be responsible (SIR) for the lowest BN content in the field 'F'. Substrate induced respiration represents the amount of C in non resting micro-organisms in soil, particularly the heterotrophs that respond to added glucose readily. Boehm and Anderson[28] observed high SIR and basal respiration in continuously cropped prairie soils than in crop – fallow soils. In this study, however, a significantly higher SIR was observed in the undisturbed forest which may indicate presence of a higher C starving population as was reflected in its high BN. Chander and Brookes[31] reported a significantly higher SIR for soils with total Cu contents of 125 mg kg^{-1} than those with 26 mg kg^{-1}.

Figure 1: Relationship between pH and DTPA extractable Cu % in experimental soils for replicates with (a) pH > 5 (n=12) and (b) pH < 5 (n=24).



Although total Cu contents of studied soils were less than 26 mg kg^{-1}, two soils from forest and field 'A', which had high total and available Cu contents resulted in a higher SIR than the rest. This observation may indicate a possible stressful condition caused by Cu on total microbial population in those soils. Negative correlations were reported between total Cu and SIR, microbial biomass, metabolic quotient, enzyme activity and bacterial biomass for soils with total Cu ranging from 20 to 400 mg kg^{-1}soil[32,33]. Such negative correlations were not established in this study because total Cu contents of experimental soils varied within a narrow range and organic C and pH also differed significantly.

The culturable fraction of bacteria was lowest in the cultivated fields 'A' and 'F' and the forest soils (Table 3). Low bacterial population reported in forest may partly be due to the presence of a larger fraction of unculturable population as well as inappropriateness of the growth medium to cultivate a starving population. Lovell *et al*.[34] also suggested that nutrient rich medium such as TSA may result in low apparent colony counts contradictorily to the high microbial activities in undisturbed soils. The lowest bacterial populations of 'A' and 'F' fields are mainly due to low organic C contents and acidic pH. In addition, high DTPA extractable Cu may also be another reason for the affected bacterial population.

The colony counts of the Cu-resistant bacteria followed the same trend to that of the total bacterial counts (Table 3). Consequently, a positive correlation was established between Cu resistant bacteria and total bacterial population ($R = 0.83$, $p < 0.001$). This correlation provides evidence that indigenous microbial communities of experimental soils are comprised of higher populations of Cu-resistant bacteria. Enumeration of Cu-resistant bacteria was done in agar plates having approximately 800 mg Cu L^{-1}. This concentration was several hundred folds higher than the Cu levels experienced by bacteria *in situ*. Consequently, the percentage of Cu-resistant bacteria populations remained lesser than 1% of the total bacterial population. Similar percentages have been reported previously for Cu–resistant bacteria extracted from manured soils in Belgium and grown on 8 mM Cu[16]. Viti *et al*. [35] reported that a forest soil having soluble + exchangeable fractions of Cu comparable to those of cultivated soils (1.3 – 4.2 mg kg^{-1} soil) did not produce any colonies on Cu amended TSB agar plates. However, forest soil of this study showed the highest percentage of Cu-resistant bacteria on TSA medium. Furthermore, fields 'A' and 'F' showed the lowest percentage of Cu-resistant bacteria (0.02% and 0.001%, respectively) despite higher Cu fractions reported (Table 3). These results may suggest that the variability of the Cu-resistant bacterial population in the studied fields is independent from the reported Cu fractions and perhaps length of exposure to Cu as well. There is evidence that Cu-tolerant bacteria populations did not relate to soluble and exchangeable fractions of Cu in cultivated soils[35]. The authors suggest that either bacterial tolerance developed responding to acute toxicity of Cu immediately after applying Cu or to the fraction bound to a more labile humic fraction. In this study, a weak correlation was established between the percentage of Cu-resistant bacteria and DTPA extractable Cu content ($R = 0.49$, $p < 0.03$, $n = 42$) suggesting that the extractable fractions of Cu reflect the range of threshold Cu levels at which resistance development takes place. It has been pointed out that high variability of microbial responses to Cu may arise as a result of higher sensitivity of micro-organisms to soil chemical and physical properties[14]. In the studied cultivated soils, pH and organic C appeared as the governing factors of the populations of Cu-resistant bacteria. These chemical properties are known to influence the fluxes of various fractions of Cu[3,7,10] Correlations were also observed between the percentage of Cu-resistant bacteria and SIR

Table 3: Biomass N, SIR and bacterial populations of experimental soils

Field	BN (μg N g^{-1} soil)	SIR (μg CO_2 g^{-1} soil h^{-1})	Bacteria (CFU X 10^7 g^{-1} Carbon)	Cu-resistant bacteria (CFU X 10^3 g^{-1} Carbon)	Cu-resistant bacteria (%)
A	173 ^{ab}	32 ^{b}	87 ^{c}	8 ^{c}	0.02 ^{b}
B	168 ^{ab}	17 ^{c}	2054 ^{a}	5224 ^{a}	0.03 ^{b}
C	152 ^{ab}	9 ^{c}	843 ^{bc}	2815 ^{b}	0.09 ^{b}
D	127 ^{ab}	14 ^{c}	352 ^{bc}	2309 ^{bc}	0.15 ^{b}
E	129 ^{abc}	8 ^{c}	1164 ^{ab}	2155 ^{bc}	0.03 ^{b}
F	58 ^{b}	10 ^{c}	36 ^{c}	3 ^{c}	0.001 ^{b}
forest	267 ^{a}	46 ^{a}	0.6 ^{c}	11 ^{c}	0.43 ^{a}

Means (n = 6) given in a column followed by different letters are significantly different (subset for $\alpha = 0.05$)

Table 4: Total and Cu-resistant bacterial populations obtained from four growth media

Agar medium	Total population (CFU X 10^7 g^{-1} carbon)	Cu resistant bacteria (CFU X 10^3 g^{-1} carbon)	Cu resistant bacteria (%)
Forest			
TSA	0.58 [b]	11 [b]	0.42 [a]
PDYA	5.34 [ab]	150 [a]	0.33 [ab]
PGA	8.62 [a]	106 [a]	0.17 [bc]
TSB	2.51 [b]	19 [b]	0.07 [c]
Field 'F'			
TSA	320 [b]	2 [b]	0.0009 [b]
PDYA	1110 [a]	1038 [a]	0.0084 [a]
PGA	1100 [a]	392 [b]	0.0031 [b]
TSB	770 [a]	17 [b]	0.0007 [b]

Means (n = 6) given in a column followed by different letters are significantly different. (subset for $\alpha = 0.05$)

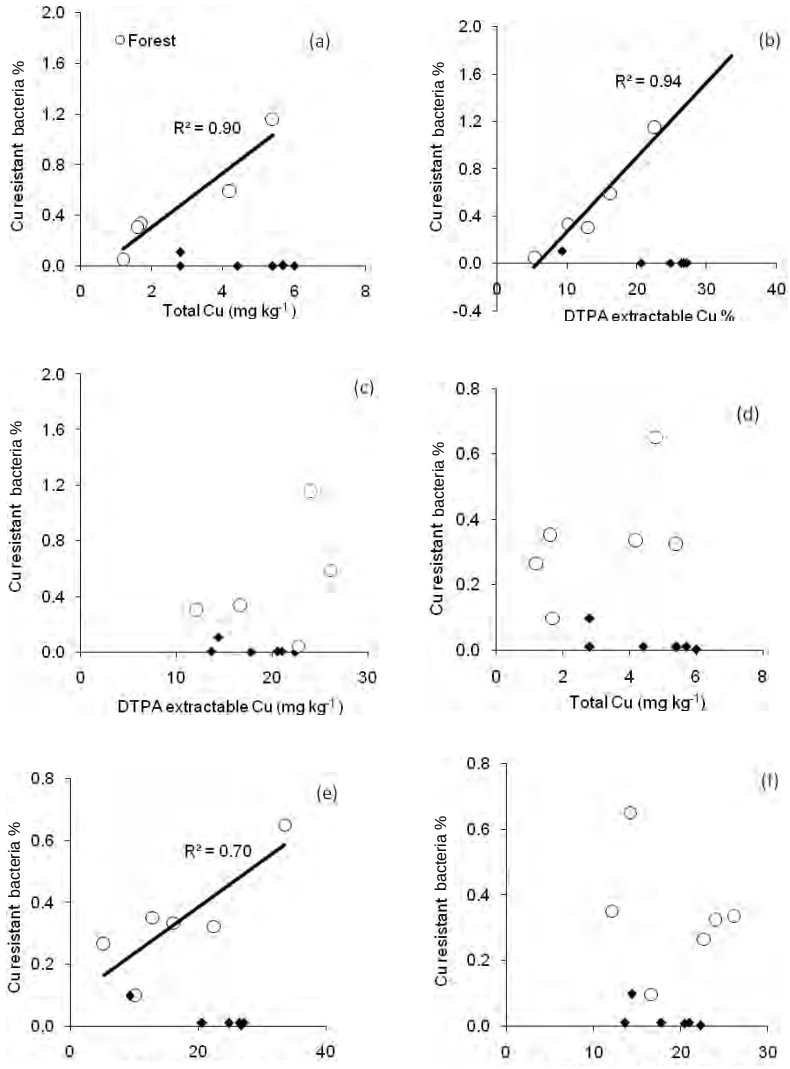


Figure 2: Relationship between Cu fractions in soil and percentage of Cu resistant bacteria (n = 6) appeared on Cu amended TSA medium (a, b and c) and PDYA medium (d, e and f)

($R = 0.48$, $p < 0.05$) and organic C ($R = 0.49$, $p < 0.05$). It is known that the available C pool supports the survival of bacteria under metal stressful conditions[32]. Accordingly, limited availability of C in cultivated fields may also have hindered the proliferation of Cu-resistant bacteria. Thus, the observed disparity between DTPA extractable Cu and Cu-resistant bacterial populations may be accounted for by the high variability in total Cu, pH and organic C between fields.

Huysman *et al*. [16] demonstrated that Cu resistance in bacteria could be developed due to long-term exposure to DTPA extractable metal contents as low as 3 mg kg^{-1}. Saek *et al*. [18] also demonstrated that bacterial resistance to Cu increased with increasing metal content from 0.011 to 12 mg kg^{-1} soil in the exchangeable fraction. Therefore, possibility of developing resistance to Cu in bacteria of the studied fields cannot be overruled although it is not supported by strong correlations between the bacterial populations and Cu fractions.

Culture media

Suitability of four growth media to enumerate metal resistant bacteria was assessed using soils from forest and field 'F', which showed the highest DTPA extractable Cu content. In both fields, populations of the total and the Cu-resistant bacteria were different between four media assessed (Table 4). It is possible to select different bacterial populations by each medium as their constituents are different. In addition, Cu availability may also differ between tested media. Ramamoorthy and Kushner[36] studied the binding of Hg^{2+}, Pb^{2+}, Cu^{2+} and Cd^{2+} ions to widely used bacterial growth media and reported that the relative affinity of different media components to different ions varied with the cation studied. Colonies on agar plates consisting of TSA, PDYA and PGA media appeared after one day of incubation, whereas it took three to four days on the TSB medium. Higher percentages of Cu-resistant bacteria were produced in PDYA and TSA plates inoculated with forest soil and on PDYA medium inoculated with the cultivated soil (Table 4). Percentage of Cu-resistant bacteria of forest soils appeared on TSA and PDYA media showed linear relationships with total Cu and percentage of DTPA extractable Cu but not with DTPA extractable Cu content in soil (Figures 2a and 2b). This may imply that Cu availability in TSA and PDYA media may represent the average Cu levels experienced by bacteria *in situ*. Since the fluctuation of Cu in the forest soil is minor, the extractable fractions of Cu at the time of sampling may represent average values that exist in soil. Accordingly, bacteria population appeared on these media may represent the population which is resistant to the reported Cu fractions. In contrast, available fractions of Cu may fluctuate temporally in the cultivated soil, along with the changes in soil pH and the organic C content. As a result, Cu-resistant bacterial populations may not essentially represent those who are resistance to the Cu fractions reported at the time of sampling. As suggested previously[35] the resistance may develop to the highest levels of Cu to which bacteria were exposed frequently. Therefore, linear relationships were not established between Cu fractions and Cu resistant bacterial populations of cultivated soils.

CONCLUSION

Resistance to Cu has developed in bacteria of the studied fields responding to the DTPA extractable Cu concentrations higher than 1.2 mg kg^{-1} soil. Confounding effect of soil pH and organic C was evident on the variability of Cu fractions and Cu-resistant bacterial populations of the cultivated fields. In addition, SIR and BN of the studied fields were sensitive to Cu. Population of Cu-resistant bacteria did not vary over the years of cultivation but correlated with the DTPA extractable Cu contents. The culturable fraction of Cu-resistant bacterial populations was enumerated by the PDYA medium better than the three other tested media. Results suggest that Cu-resistant bacterial population could be treated as an indicator of soil quality of the studied fields if interpreted in conjunction with the variability of pH and organic C.

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