Research Article

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Copper tolerance and antibiotic resistance in soil bacteria from olive tree agricultural fields routinely treated with copper compounds

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Abstract

BACKGROUND: Heavy metal pollution may act as persistent selective pressure that favors the spread of antimicrobial resistance in natural environments. The aim of this study was to isolate and identify metal-tolerant bacteria from soils in olive tree fields routinely treated with copper-derived compounds and to evaluate the tolerance of bacterial strains to other metals and their resistance to clinically relevant antibiotics.

RESULTS: Five hundred and ninety-five bacterial isolates from 45 olive tree agricultural fields were studied. Minimum inhibitory concentrations (MICs) $\geq$ 16 mmol L^{-1} were detected for copper (57% of isolates), zinc (37%) and lead (62%), while only 3% had MICs $\geq$ 12 mmol L^{-1} for nickel. Ninety-six metal-tolerant strains were selected for identification and antibiotic resistance determination. Most isolates belonged to the genera *Pseudomonas* (37%), *Bacillus* (23%) and *Chryseobacterium* (20%), while 6% were identified as *Variovorax*, 4% as *Stenotrophomonas* and 2% as *Serratia* or *Burkholderia*. Highest copper tolerance was detected among *Pseudomonas*. Over 75% of the strains with high copper tolerance were also resistant to vancomycin, 50% to ampicillin and 40% to erythromycin or trimethoprim/sulfamethoxazole.

CONCLUSION: Bacteria from olive soils are tolerant to metals, mainly copper, but also zinc and lead, as well as resistant to clinically important antibiotics, which could be a troublesome issue in clinical settings.

Supporting information may be found in the online version of this article.

Keywords: copper tolerance; metal tolerance; antibiotic resistance; olive tree fields

INTRODUCTION

Because of their toxic effects, heavy metals can induce remarkable modifications of the microbial communities in the environment as well as changes in microbial activities.[1] In recent years, scientific evidence has proved conclusively that heavy metal pollution may act as an agent of persistent selective pressure that favors the spread of antimicrobial resistance in natural environments.[2,3] Antibiotic resistance is recognized as a major global threat to public health by the World Health Organization. Currently, several hundred thousand deaths yearly can be attributed to infections with antibiotic-resistant bacteria, and different studies have corroborated that soil contamination with heavy metals[4] and in particular copper[5,6] may increase bacterial resistance to antibiotics. Non-antibiotic compounds such as metals may contribute to the promotion of antibiotic resistance through co-selection[7] or counter-selection.[8] Co-selection implies that exposure to a heavy metal results in maintenance of antibiotic resistance traits in bacteria.[2] Available data suggest that there are different mechanisms involved in co-selection of resistance, although mainly gene transfer activities have been described as responsible.[9]

Indeed, there is a growing body of evidence for Cu/Zn driving antibiotic resistance development in metal-exposed bacteria owing to metal selection of genetic elements harboring both metal and antibiotic resistance genes and metal recruitment of antibiotic resistance mechanisms.[10] There are clearly established links between the resistance genes found in soil and animals and resistance genes found in humans, including human pathogenic bacteria, with antibiotic resistance genes found in the former also present in human pathogens.[11,12] These links have been seen in diverse environments that include industrial,[13,14] clinical,[15] aquatic[13,16] (including wastewater)[17] and, especially, agricultural[18–22] settings.

Most previous studies on co-selection of heavy metal and antibiotic resistances have focused on soils and waste near industrial areas contaminated with wastewater sediments.[2,23] However, there is growing concern that agricultural treatments with heavy metal-derived salts may also be relevant for the selection of drug-resistant bacteria in soils.[24,25]

The total Cu concentration in soil depends on the type and mineral properties of the parent material as well as the process by which the soil was formed.[25] In addition, anthropogenic activities

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such as agriculture, industrialization, mining, traffic and domestic heating can make a significant contribution to the accumulation of Cu in soil.[26] For instance, the use of copper sulfate (Bordeaux mixture) as a fungicide against mildew in the viticulture sector has resulted in the Cu contamination of vineyard soils at levels ranging from 100 to 1500 mg kg^{-1}.[27]

Because of the extended use of Cu-derived compounds as antifungals in olive tree agricultural fields in the province of Jaén, this metal is expected to be the main one responsible for the propagation of antibiotic resistance in this environment, as previously established.[15] The aim of the present study was to isolate and identify metal-tolerant bacteria from soils in olive tree fields routinely treated with Cu-derived compounds. The tolerance of these bacterial strains to other metals and their resistance to clinically relevant antibiotics were also evaluated in order to ascertain if the contact with Cu salts may favor the spread of metal tolerance and/or antibiotic resistance in this environment.

The purpose of this study was to learn more about the co-occurrence of antibiotic-resistant and heavy metal-tolerant bacteria in soils from olive tree fields.

MATERIALS AND METHODS

Sample collection and determination of Cu concentration in soil

Soil samples were collected from December 2013 to February 2014 from 45 olive tree agricultural fields randomly selected throughout the province of Jaén, Andalusia, Spain. Geographic locations of the sampling sites are shown in Fig. S1 and geographic coordinates are summarized in Table S1.

Samples were collected from a depth of 5–10 cm of the superficial layer according to Menjivar *et al.*[28] and transported to the laboratory under refrigeration in sterile plastic bags for bacteriological analysis.

Copper concentrations in dry soil samples were analyzed after extraction with diethylenetriamine pentaacetic acid (DTPA) according to Lindsay and Norvell.[29] All reagents were purchased from Sigma-Aldrich (Madrid, Spain). Extracting solution (0.01 mol L^{-1} $CaCl_2$, 0.005 mol L^{-1} DTPA and 0.1 mol L^{-1} triethanolamine) was adjusted to pH 7.3 with 1 mol L^{-1} HCl. Samples (10 g) were added to 20 mL of extracting solution and shaken (120 rpm) for 2 h. Suspensions were filtered (Whatman grade 42 filter paper) and Cu concentration was determined with a spectrometer (Perkin Elmer Optima 8000, Waltham, MA, USA) by inductively coupled plasma optical emission spectrometry (ICP-OES).

Bacterial isolation and purification

Stock solutions of metal salts (1 mol L^{-1} in distilled water) were filter-sterilized (0.22 µm) and stored at 4 °C. Soil samples (0.5 g) were inoculated into flasks containing 4.5 mL of Luria Bertani (LB) medium (Scharlau, Barcelona, Spain) and incubated for 1 h at 30 °C with shaking (180 rpm). Soil particles were allowed to settle for 30 min. Then the liquid phase was serially diluted five times and spread-plated on LB agar (Scharlau) added with different concentrations of metal salts (all from PanReac, Barcelona, Spain) as previously described by Icgen and Yilmaz,[2] with slight modification: $Pb(NO_3)_2$ (600 mg L^{-1}, 1.8 mmol L^{-1}), $CuSO_4{\cdot}5H_2O$ (450 mg L^{-1}, 1.8 mmol L^{-1}), $NiSO_4{\cdot}6H_2O$ (395 mg L^{-1}, 1.5 mmol L^{-1}), $ZnSO_4{\cdot}7H_2O$ (825 mg L^{-1}, 2.86 mmol L^{-1}), $Cd(NO_3)_2{\cdot}4H_2O$ (80 mg L^{-1}, 0.26 mmol L^{-1}), pH 7.0. Plates were incubated at 30 °C and inspected for bacterial growth for 5 days. Up to five colonies with different morphological appearance were isolated from each plate, as they were perceptible, for subsequent analysis. A panel of 595 isolates representative of the 45 soil samples was purified by repeated streaking on LB agar plates and selected for further study. Stocks of isolates in LB supplemented with 20% (v/v) glycerol were stored at −80 °C.

Determination of bacterial tolerance to metals

Stock solutions of metal salts previously described were added to LB agar plates to reach the concentrations specified in Table 2: Cu, 2–32 mmol L^{-1} (127.1–2034 mg L^{-1}); Cd, 0.5–16 mmol L^{-1} (56.21–1798.56 mg L^{-1}); Pb, 4–32 mmol L^{-1} (828.8–6630.4 mg L^{-1}); Zn, 4–32 mmol L^{-1} (261.56–2092.48 mg L^{-1}); Ni, 4–32 mmol L^{-1} (234.76–1878.08 mg L^{-1}). Then the plates were inoculated with 5 µL (10^7 colony-forming units (CFU) mL^{-1}) from 10-fold dilutions in sterile saline solution of overnight cultures of the 595 bacterial isolates and incubated for 24–48 h at 30 °C. The lowest metal concentration that inhibited bacterial growth was taken as the minimum inhibitory concentration (MIC).

Strain identification

Ninety-six strains were selected for further studies based on their high tolerance to at least three of the metal salts analyzed, and covering the different sampling areas. Selected strains were identified by 16S rDNA sequencing. Following DNA extraction (GenElute™ Gel Extraction Kit, Sigma-Aldrich), 16S rDNA was amplified as described by Abriouel *et al.*[30] Polymerase chain reaction (PCR) amplification products were purified using a GFX PCR DNA and Gel Band Purification Kit (GE-Healthcare, Madrid, Spain) and then sequenced according to Weisburg *et al.*[31] in a CEQ 2000 XL DNA Analysis System (Beckman Coulter, West Sacramento, CA, USA). The DNA sequences obtained were searched for homology by using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

Determination of antibiotic resistance

Antibiotic resistance of the selected strains was determined by disk diffusion following CLSI indications and group-dependent breakpoints (Clinical and Laboratory Standards Institute[32]). Mueller Hinton agar (cation-adjusted) (Fluka, Sigma-Aldrich) and disks with clinically important antibiotics from Oxoid (Basingstoke, UK) were used: ampicillin (AMP, 10 µg), ceftazidime (CAZ, 30 µg), gentamicin (CN, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), vancomycin (VA, 30 µg) and trimethoprim/sulfamethoxazole 1:19 (co-trimoxazole) (STX, 25 µg). The antimicrobial activity was expressed in terms of the average diameter (mm) of the zone of inhibited growth around the disks.

Statistics

Correspondence analysis was performed to define the relationships between metal tolerances and bacterial identification as well as between Cu tolerance and resistance to antibiotics. Chi-square distances were calculated to display contributions of each cell to the chi-square statistic used to test the hypothesis of independence between row and column classifications (R: a Language and Environment for Statistical Computing, R Foundation for Statistical Computing, R Core Team, 2017, Vienna, Austria).

Principal component analysis (PCA) was used to analyze relationships among Cu concentration in soil samples and tolerance to Cu in bacterial isolates, as well as among different metal tolerances (Statgraphics Centurion XVI, version 16.2.04, 2013, EEUU).

Table 1. Distribution of soil samples according to Cu concentration

Cu (mg kg^{-1})	2–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–70	75–80	81–120	125–130
Soil samples (%)	26.67	31.11	20.00	4.44	6.67	0	2.22	4.44	0	2.22	0	2.22

Table 2. Distribution of 595 selected isolates (%) according to their minimum inhibitory concentration (MIC) of metal salts

MIC (mmol L^{-1})	$CuSO_4 \cdot 5H_2O$	$ZnSO_4 \cdot 7H_2O$	$Pb(NO_3)_2$	$Cd(NO_3)_2 \cdot 4H_2O$	$NiSO_4 \cdot 6H_2O$
0.5				3.03	
1				53.95	
2	4.03	33.11		19.33	
4	9.92	19.50	2.02	9.24	44.37
8	10.76	9.75	14.62	14.45	52.44
12	18.66		21.01		0.17
16	27.56	30.76	61.34		3.03
20	10.42				
32	18.66	6.89	1.01		

RESULTS

Copper concentration in soil

Copper concentrations in soil samples (Table S1) ranged from 2 to 129 mg kg^{-1}. The average Cu concentration was 15.22 mg kg^{-1}; 57.8% of samples showed Cu concentrations between 2 and 10 mg kg^{-1} and 77.78% showed Cu concentrations $\leq$ 15 mg kg^{-1} (Table 1). Only two soil samples (T36 and T23) had remarkably high Cu concentrations of about 78 and 129 mg kg^{-1} respectively.

Metal tolerance in bacterial isolates

Results on metal tolerance of the 595 isolates used in the study are shown in Table 2. Fifty-six per cent of isolates showed MICs $\geq$ 16 mmol L^{-1} for copper sulfate, and still 18.7% needed a concentration of 32 mmol L^{-1} copper sulfate to be inhibited. High tolerances to zinc sulfate (37% of isolates displaying MICs $\geq$ 16 mmol L^{-1}) and lead nitrate (62% of isolates showing MICs $\geq$ 16 mmol L^{-1}) were also found. Twenty-three per cent of isolates also showed MICs $\geq$ 4 mmol L^{-1} for cadmium nitrate. Most isolates were inhibited at 8 mmol L^{-1} nickel sulfate, and only 3% of isolates were able to grow at $\geq$12 mmol L^{-1} of this metal salt.

Analysis of the cross-distribution of MICs among different metals (Fig. S2) indicates that isolates with higher MICs for a given metal did not show higher MICs for the other metals. However, there were some exceptions for lower MIC values. For example, comparing Cu and Pb (Fig. S2B), many of the isolates with Cu MICs of 12 mmol L^{-1} (95.5%), 16 mmol L^{-1} (46.3%), 20 mmol L^{-1} (71%) or 32 mmol L^{-1} (67.6%) also had a high MIC of 16 mmol L^{-1} for Pb. Comparing Cu and Ni, 65.8% of isolates having a Cu MIC of 12 mmol L^{-1} and 75.6% of those having a Cu MIC of 16 mmol L^{-1} also had a high MIC of 8 mmol L^{-1} for Ni (Fig. S2C). For Cu and Zn, 48% of isolates having a Cu MIC of 16 mmol L^{-1} also had high MIC for Zn (16 mmol L^{-1}) (Fig. S2D).

Comparison of the percentages of strains with high MICs for Cu and Cu concentrations in soil (Table S2) indicated that only the soil with a Cu concentration of 77.8 mg kg^{-1} had a higher percentage of strains with Cu MICs $\geq$ 16 mmol L^{-1} (73.3% compared with 44.4–56.3% for the rest of the soils).

Characterization of metal-tolerant isolates

A total of 96 metal-tolerant strains from 45 samples of olive tree agricultural soils were selected for further studies based on their high tolerance to at least three of the metal salts analyzed, and covering the different sampling areas (Table 3). According to results from 16S rDNA sequencing, most isolates belonged to the genera *Pseudomonas* (37.5%), *Bacillus* (22.92%) and *Chryseobacterium* (19.79%). The rest of the isolates belonged to *Variovorax* (6.25%), *Stenotrophomonas* (4.17%), *Serratia*, *Burkholderia*, *Enterobacter* and *Enterococcus* (2.08% each) or *Janthinobacterium* and *Sphingobacterium* (1% each).

Table 3 shows the tolerance to heavy metal salts and the resistance to antibiotics in selected bacterial isolates from olive tree agricultural soils. The term 'resistance' is used to describe the ability of microorganisms to grow at high concentrations of an antibiotic, irrespective of the duration of treatment, and is quantified by the MIC of the particular antibiotic, whereas 'tolerance' is more generally used to describe the ability, whether inherited or not, of microorganisms to survive transient exposure to high concentrations of a non-antibiotic compound, such as biocides or metals, without interpretative criteria available in the current CLSI documents that make it possible to clearly categorize the strains as resistant or susceptible.[33]

Analysis of the MICs of metals for the 96 selected isolates indicated that 59% of them had MICs $\geq$ 16 mmol L^{-1} for Cu and 59.38% for Zn (Table 3). Eighty per cent of the isolates had an MIC of 16 mmol L^{-1} for Pb and 25% had an MIC of 8 mmol L^{-1} for Cd, but only 7.29% had MICs $\geq$ 12 mmol L^{-1} for Ni.

Among the isolates that displayed highest MICs for Cu (32 mmol L^{-1}), 22.3% were identified as *Pseudomonas* (Fig. 1). *Pseudomonas* was the bacterial group with higher tolerance to Cu, since 91.67% of isolates had MICs $\geq$ 20 mmol L^{-1} and still 58.33% had an MIC of 32 mmol L^{-1}. In addition, 83.33% had an MIC of 16 mmol L^{-1} for Pb. Nevertheless, only a low percentage (8.33%) of the isolates from this bacterial group had MICs for Zn $\geq$ 20 mmol L^{-1}. Most *Pseudomonas* isolates were inhibited by low concentrations of Cd (1 mmol L^{-1}), but 19.33% were more tolerant (MIC 8 mmol L^{-1}).

None of the *Bacillus* isolates had MICs > 16 mmol L^{-1} for Cu (Fig. 1). A high percentage of isolates from this group had MICs of 16 mmol L^{-1} for Cu (59.09%), Zn (59.09%) and Pb (63.63%), and only two isolates had MICs > 16 mmol L^{-1} for Zn and one for Pb. *Bacillus* isolates were inhibited at 1 or 2 mmol L^{-1} Cd and 8 mmol L^{-1} Ni. *Chryseobacterium* isolates were inhibited by lower

Table 3. Tolerance to heavy metal salts and resistance to antibiotics in 96 metal-tolerant selected bacterial isolates from olive tree agricultural soils

Species (isolate)	MIC (mmol L^{-1})					Resistance phenotype
	$CuSO_4$	$ZnSO_4$	$Pb(NO_3)_2$	$Cd(NO_3)_2$	$NiSO_4$	
Bacillus cereus (T22Pb3)	16	16	16	1	8	CAZ, SXT, AMP
Bacillus endophyticus (T37Ni3)	4	4	12	1	8	CAZ
Bacillus fordii (T11Ni1)	4	4	12	1	8	CAZ
Bacillus fordii (T18Ni1)	8	4	8	1	8	CAZ, SXT
Bacillus megaterium (T1Ni2)	4	4	8	1	8	CAZ
Bacillus psychrosaccharolyticus (T4Ni3)	8	4	8	1	8	CAZ, VA
Bacillus sp. (T17Ni3)	12	20	12	2	8	CAZ
Bacillus sp. (T19Pb3)	16	16	16	1	8	AMP
Bacillus sp. (T20Pb2)	8	8	16	1	8	AMP
Bacillus sp. (T21Zn1)	12	32	20	1	8	CAZ
Bacillus sp. (T28Pb3)	16	16	16	2	8	CAZ, AMP
Bacillus sp. (T11Pb1)	16	16	16	1	8	CAZ, AMP
Bacillus sp. (T12Pb1)	16	16	16	1	8	CAZ, AMP
Bacillus sp. (T13Ni2)	16	16	16	1	8	CAZ, SXT, AMP
Bacillus sp. (T13Pb1)	16	16	16	2	8	CAZ, SXT, AMP
Bacillus sp. (T14Pb1)	16	16	16	2	8	CAZ, AMP
Bacillus sp. (T26Ni3)	16	16	16	1	8	CAZ
Bacillus sp. (T26Pb2)	16	16	16	1	8	CAZ, AMP
Bacillus sp. (T37Ni2)	4	8	12	1	8	CAZ
Bacillus sp. (T8Pb1)	16	16	16	2	8	CAZ, SXT, AMP
Bacillus sp. (T9Ni2)	16	16	16	2	8	CAZ, SXT, AMP
Bacillus sp. (T9Pb1)	16	16	16	2	8	CAZ, SXT, AMP
Burkholderia zhejiangensis (T37Cd2)	32	8	16	8	12	E, SXT, VA, AMP
Burkholderia zhejiangensis (T38Zn3)	32	32	16	1	8	E, SXT, VA, AMP
Chryseobacterium formosense (T11Zn1)	12	32	16	1	8	CN
Chryseobacterium gleum (T14Cd2)	4	8	16	4	8	CN, CAZ, SXT, AMP
Chryseobacterium gleum (T4Ni2)	4	12	20	1	8	CN, CAZ
Chryseobacterium gleum (T6Pb1)	4	20	16	8	8	CN, AMP
Chryseobacterium gleum (T6Zn3)	12	32	16	4	8	CN, AMP
Chryseobacterium hispalense (T3Zn3)	12	32	16	8	8	CN, AMP
Chryseobacterium oranimense (E5Zn1)	12	32	16	8	8	CN, CAZ, SXT, VA, AMP
Chryseobacterium oranimense (T18Cd1)	4	16	16	8	8	CN, CAZ, VA
Chryseobacterium oranimense (T30Zn1)	12	32	16	8	8	CN, AMP
Chryseobacterium oranimense (T31Zn1)	12	32	16	4	8	CN, AMP
Chryseobacterium oranimense (T37Cd3)	4	16	16	8	12	CN, AMP
Chryseobacterium oranimense (T3Cd1)	4	16	16	8	8	AMP
Chryseobacterium oranimense (T3Ni2)	4	8	16	8	8	CN, AMP
Chryseobacterium oranimense (T3Zn2)	12	32	20	8	8	CN, AMP
Chryseobacterium oranimense (T4Cd1)	4	16	16	8	8	CN, AMP
Chryseobacterium oranimense (T5Cd1)	4	16	16	8	8	CN, AMP
Chryseobacterium oranimense (T5Ni3)	4	20	16	8	8	E, AMP
Chryseobacterium oranimense (T6Cd1)	4	16	16	8	8	CN, AMP
Chryseobacterium piperi (T8Cd1)	4	16	16	4	8	VA, AMP
Enterobacter aerogenes (E1Pb3)	16	8	16	1	8	E, VA, AMP
Enterococcus faecalis (T26Zn2)	12	32	16	1	8	E, CN, CAZ, CIP
Flavobacterium johnsoniae (E1Zn1)	12	32	16	2	16	CN, CAZ, VA
Janthinobacterium lividum (T25Cd3)	26	8	16	2	8	CAZ, SXT
Pseudomonas entomophila (E1Cu3)	20	4	16	1	8	E, CIP, SXT, VA, AMP
Pseudomonas entomophila (T11Cd1)	32	4	16	4	8	E, VA, AMP
Pseudomonas entomophila (T17Pb1)	16	4	16	1	4	E, SXT, VA, AMP,
Pseudomonas entomophila (T23Cu2)	32	4	16	0.5	4	E, SXT, VA, AMP
Pseudomonas entomophila (T34Cu2)	32	4	16	1	8	E, SXT, VA, AMP
Pseudomonas entomophila (T5Cu2)	32	8	16	1	8	E, CN, SXT, VA, AMP
Pseudomonas entomophila (T6Cu1)	32	4	16	1	4	SXT, VA, AMP
Pseudomonas entomophila (T7Ni1)	32	20	12	4	8	VA, AMP
Pseudomonas fluorescens (E5Zn2)	32	32	16	4	8	E, VA, AMP

Table 3. (*Continued*)

Species (isolate)	MIC (mmol L^{-1})					Resistance phenotype
	$CuSO_4$	$ZnSO_4$	$Pb(NO_3)_2$	$Cd(NO_3)_2$	$NiSO_4$	
Pseudomonas fluorescens (E5Zn3)	32	32	16	8	8	E, VA, AMP
Pseudomonas fluorescens (T12Cu1)	32	16	16	1	8	E, VA, AMP
Pseudomonas fluorescens (T15Cd1)	32	16	16	8	8	E, VA, AMP
Pseudomonas fluorescens (T25Cu3)	20	8	16	1	4	E, CIP, SXT, VA, AMP
Pseudomonas fluorescens (T25Ni3)	20	8	12	1	8	SXT, VA, AMP
Pseudomonas fluorescens (T31Cd3)	26	16	16	8	8	E, SXT, VA, AMP
Pseudomonas fluorescens (T32Cd3)	32	16	16	8	8	CAZ, VA, AMP
Pseudomonas fluorescens (T35Cd3)	32	16	16	8	8	E, VA, AMP
Pseudomonas fluorescens (T35Cu2)	32	8	16	1	8	E, SXT, VA, AMP
Pseudomonas fluorescens (T3Ni3)	16	4	16	1	8	E, SXT, VA, AMP
Pseudomonas fluorescens (T5Cd2)	32	8	16	8	8	E, SXT, VA, AMP
Pseudomonas fluorescens (T7Cd3)	32	16	16	8	8	E, CAZ, SXT, VA, AMP
Pseudomonas lutea (E3Cu2)	20	4	16	1	4	E, SXT, VA, AMP
Pseudomonas lutea (T8Ni2)	4	4	4	1	8	E, SXT, VA, AMP
Pseudomonas putida (T1Cu3)	32	8	16	1	4	E, SXT, VA, AMP
Pseudomonas sp. (T15Cu3)	32	4	16	1	4	E, CIP, SXT, VA, AMP
Pseudomonas sp. (T15Ni1)	16	4	12	2	8	SXT, VA, AMP
Pseudomonas sp. (T16Ni3)	16	4	12	1	8	SXT, VA
Pseudomonas sp. (T18Cu3)	20	4	16	1	4	E, CIP, SXT, VA, AMP
Pseudomonas sp. (T19Cu2)	20	4	16	2	8	E, CAZ, SXT, VA, AMP
Pseudomonas sp. (T22Cu1)	20	16	16	2	8	E, SXT, VA, AMP
Pseudomonas sp. (T24Cu3)	32	4	16	1	4	E, SXT, VA, AMP
Pseudomonas sp. (T24Ni1)	8	4	8	1	8	CAZ
Pseudomonas sp. (T28Cu2)	32	4	16	1	8	E, SXT, VA, AMP
Pseudomonas sp. (T36Cu3)	20	4	16	1	8	E, CAZ, SXT, VA, AMP
Pseudomonas sp. (T40Cd3)	32	16	16	4	8	E, CAZ, SXT, VA, AMP
Pseudomonas sp. (T7Cu2)	32	4	16	4	4	E, SXT, VA, AMP
Serratia proteamaculans (T3Cd2)	32	8	16	8	8	VA, AMP
Serratia proteamaculans (T4Pb1)	20	16	16	8	8	VA, AMP
Sphingobacterium paucimobilis (T30Zn3)	12	32	16	8	16	E, CN
Stenotrophomonas maltophilia (T19Zn1)	12	32	20	4	16	E, CN, SXT, AMP
Stenotrophomonas maltophilia (T40Zn1)	12	32	16	4	16	VA, AMP
Stenotrophomonas rhizophila (E1Zn3)	12	32	16	0.5	4	E
Stenotrophomonas rhizophila (T31Zn2)	12	32	16	1	8	AMP
Variovorax paradoxus (E4Zn1)	16	32	16	4	4	E, CN, CAZ, SXT, VA, AMP
Variovorax paradoxus (T18Zn2)	12	32	8	2	4	CN, CAZ, VA, AMP
Variovorax paradoxus (T23Zn2)	12	32	16	4	8	CN, CAZ, SXT, VA, AMP
Variovorax paradoxus (T32Zn2)	16	32	16	2	16	E, CN, CAZ, VA, AMP
Variovorax paradoxus (T32Zn3)	16	32	16	2	8	CN, CAZ, VA, AMP
Variovorax paradoxus (T7Zn1)	16	32	8	4	4	E, CN, CAZ, VA, AMP

AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; E, erythromycin; SXT, trimethoprim/sulfamethoxazole; VA, vancomycin; MIC, minimum inhibitory concentration.

Cu concentrations of 4 or 12 mmol L^{-1} (Fig. 1). However, 42.1% only were inhibited at Zn concentrations of 20 mmol L^{-1} or higher. Most *Chryseobacterium* isolates were inhibited by 16 mmol L^{-1} Pb (89.47%) and 8 mmol L^{-1} Ni (94.73%), but 68.42% required 8 mmol L^{-1} Cd for inhibition.

Among the 96 isolates studied, antibiotic resistance (Table 3) was most frequent for AMP (80.21%) followed by VA (54.16%), STX (43.71%), E (41.66%), CAZ (40.63%), CN (28.12%) and CIP (5.20%). For the genus *Bacillus*, highest percentages of resistances were obtained for CAZ (86.36%) followed by AMP (54.55%), STX (27.27%) and VA (4.55%); for *Chryseobacterium*, AMP and CN (84.21%), CAZ (21.05%), VA (15.79%), STX (10.53%) and E (5.27%); for *Pseudomonas*, VA (97.22%), AMP (94.44%), E (80.56%), STX (75%), CAZ (16.67%), CIP (11.11%) and CN (2.78%). Although the genus *Variovorax* was represented by only five isolates, it is worth mentioning that all of them were resistant to CAZ, AMP, CN and VA.

Correspondence analysis performed to define the relationships between Cu tolerance and resistance to antibiotics (Fig. 2) shows high associations between tolerance to this metal and resistance to clinically important antibiotics such as ampicillin (Fig. 2(a)), vancomycin (Fig. 2(b)), erythromycin (Fig. 2(c)) and the combination trimethoprim/sulfamethoxazole (Fig. 2(e)). Over 75% of the Cu-tolerant selected strains were also resistant to vancomycin, 50% to ampicillin and 40% to erythromycin or

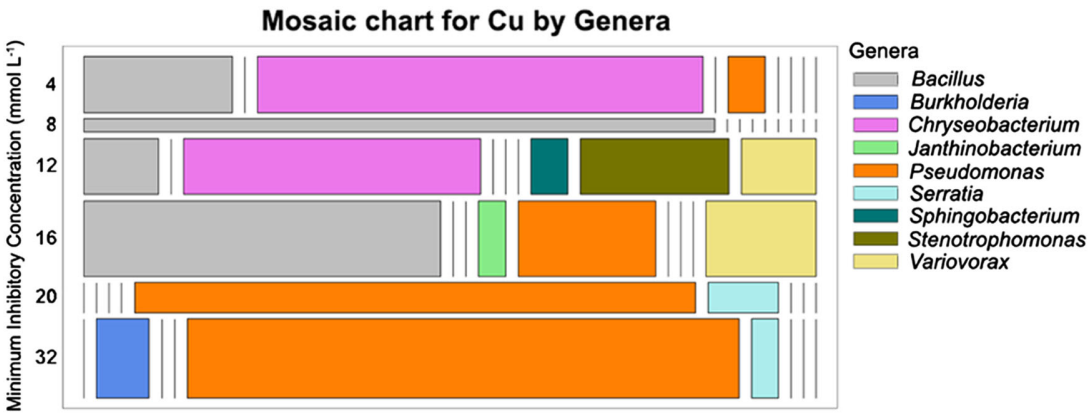

Figure 1. Distribution of Cu tolerance (mmol L^{-1}) according to identified genera.

trimethoprim/sulfamethoxazole ($P < 0.05$), showing strong correlations. Over 35% of all these antibiotic-resistant strains showed MICs of 20–32 mmol L^{-1} for copper sulfate, and in addition over 31% belonged to the genus *Pseudomonas*.

Thirty per cent of the Cu-tolerant strains were also resistant to ceftazidime (Fig. 2(d)) or gentamicin (Fig. 2(f)). However, in both cases, antibiotic-resistant strains showed MICs for copper sulfate ranging between 4 and 32 mmol L^{-1}, which indicates no correlation between metal tolerance and antibiotic resistance. On the other hand, most Cu-tolerant strains were inhibited by ciprofloxacin (Fig. 2(g)).

DISCUSSION

Copper-derived compounds are usually spread in olive tree agricultural fields in the province of Jaén, Andalusia, Spain to prevent or treat fungal infections. Owing to its prolonged persistence in soil,[24] Cu may pose long-standing selective pressure for antimicrobial resistance.[34,35]

There is a long history of metals, including Cu and Zn, being linked to antibiotic resistance development in environmental bacteria, with many studies showing a correlation between the presence of either of these two metals in environmental samples and the concomitant presence of metal-tolerant and antibiotic-resistant populations, or the increased prevalence of antibiotic-resistant genes/organisms in metal-contaminated *versus* metal-free environments.[18,19,36] Moreover, the impact of Cu on antimicrobial resistance is higher compared with other metals.[37,38]

According to results from the present study, most olive farm soils had low Cu concentrations with an average of 15 mg kg^{-1} (which would be equivalent to approximately between 0.2 and 0.3 mmol L^{-1} Cu) and only two soils had higher concentrations of 78 mg kg^{-1} (*ca* 1.1–1.4 mmol L^{-1}) and 129 mg kg^{-1} (*ca* 1.8–2.4 mmol L^{-1}). However, the percentages of isolates with Cu MICs $\geq$ 16 mmol L^{-1} were very high, reaching up to 32 mmol L^{-1}. These results could be interpreted by several factors. For example, episodes of exposure to high Cu concentrations could select for bacteria with higher Cu tolerance that then would persist in the soil after the Cu concentration decreased. In second place, if Cu is not distributed homogeneously in the soil, the presence of microenvironments (such as soil particles) with higher Cu concentration could create selective pressure for Cu tolerance. There is a growing body of evidence for Cu/Zn driving antibiotic resistance development in metal-exposed bacteria owing to metal selection of genetic elements harboring both metal and antibiotic resistance genes and metal recruitment of antibiotic resistance mechanisms.[10] The repeated addition of Cu also causes a higher tolerance to other compounds than that in a single addition, suggesting that repeated exposure mitigates the toxicity and promotes the selection for tolerant bacteria.[39] Therefore it is also possible that repeated exposure to low concentrations of Cu would induce high levels of tolerance to this metal. Furthermore, relatively high percentages of the bacteria isolated from olive farm soils also had high levels of tolerance to other metals such as Zn and Pb, and in lower percentages also to Cd and Ni. These results would also suggest naturally existing populations of metal-tolerant bacteria in the soils and common mechanisms of tolerance to these metals, such as those based on non-specific adsorption/immobilization of the metal.

Combined tolerance to heavy metals has also been described by Silver and Ji,[40] as well as co-selection of tolerance to metals and to antibiotics in heavy metal-polluted samples,[24,41,42] probably as a result of gene transfer activities among bacteria from different sources. Results from the present study are of concern as they reveal the presence of phenotypic antibiotic resistance in bacterial isolates tolerant to metals from olive farm soils. In particular, high percentages of isolates with high MICs for Cu also were resistant to antibiotics such as ampicillin, vancomycin, erythromycin and trimethoprim/sulfamethoxazole. Previous studies corroborate antimicrobial resistance in bacterial populations from soils and its association with tolerance to heavy metals in agriculture production.[5,43]

Our results also indicate the high presence of metal-resistant *Pseudomonas* in olive farm soils, as previously reported in other heavy metal-polluted soils,[9,44] and describe its association with antibiotic resistance.

The global priority pathogens list (global PPL) of antibiotic-resistant bacteria recently published by the World Health Organization, as requested by Member States to help in prioritizing the research and development (R&D) of new and effective antibiotic treatments, points to the genera *Acinetobacter* and *Pseudomonas*, specifically the carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains, within the group Priority 1: CRITICAL for R&D of new antibiotics.

Multidrug-resistant *Pseudomonas* strains are disseminated worldwide[45] and may cause infections with high mortality rates among debilitated persons.[46] Environmental bacteria may be involved in opportunistic infections that may be of fatal consequence if the bacteria are antibiotic-resistant (regardless of whether they have intrinsic or acquired resistance). Remarkably,

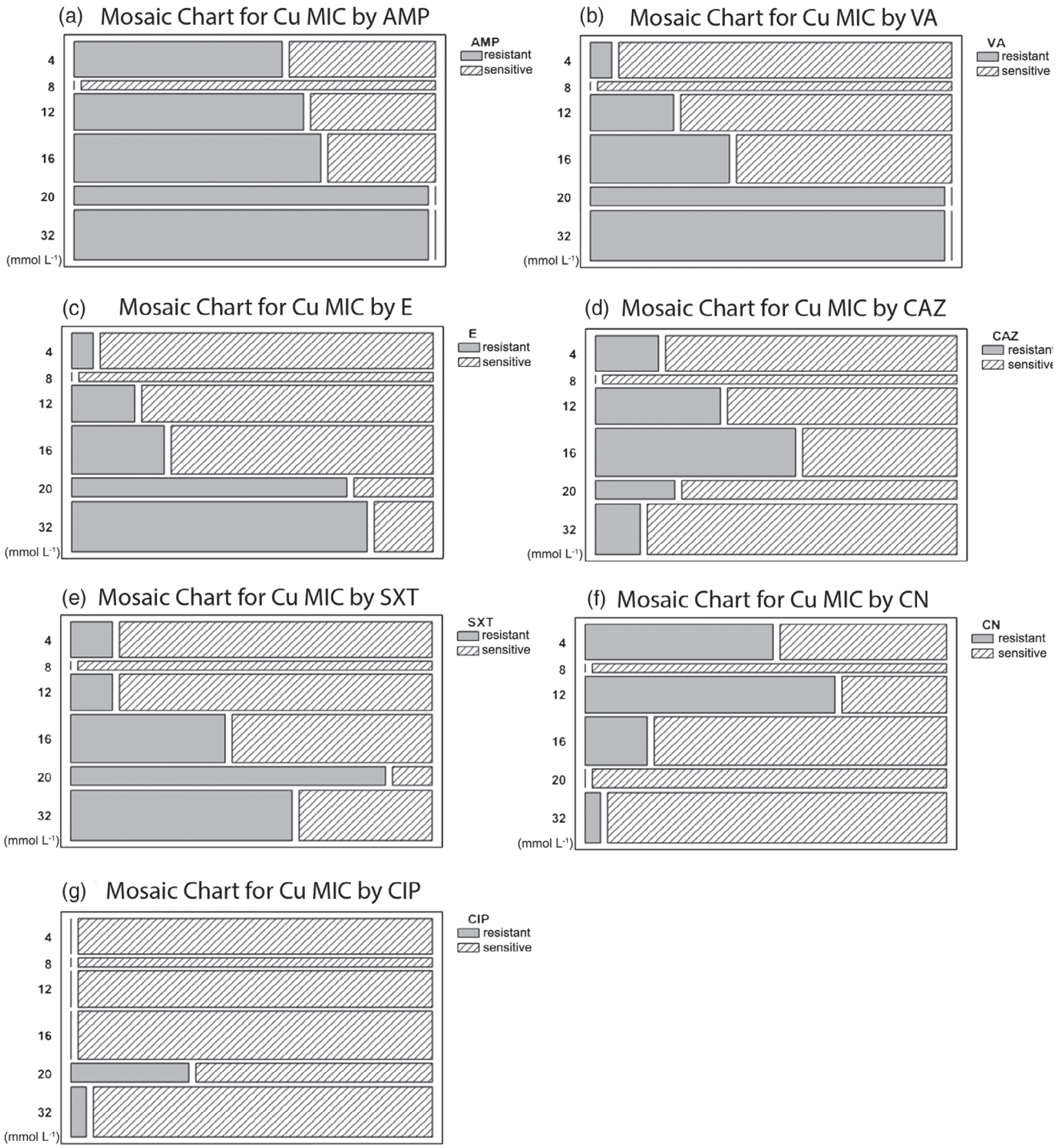

Figure 2. Distribution of resistance to antibiotics according to Cu tolerance (mmol L^{-1}): (a) ampicillin; (b) vancomycin; (c) erythromycin; (d) ceftazidime; (e) trimethoprim/sulfamethoxazole; (f) gentamicin; (g) ciprofloxacin.

nine of the *Pseudomonas* isolates from the present study were resistant to five antibiotics.

CONCLUSIONS

Results from the present study show high percentages of isolates with Cu MICs $\geq$ 16 mmol L^{-1}, although most olive farm soils have low Cu concentrations. Furthermore, relatively high percentages of the bacteria isolated from olive farm soils also had high levels of tolerance to other metals such as Zn and Pb, and in lower percentages also to Cd and Ni. These results would suggest naturally existing populations of metal-tolerant bacteria in the soils.

As antibiotic resistance was frequently found in these metal-tolerant bacteria, further studies on the genetic determinants of antibiotic resistance will be needed to establish the potential of the isolated metal-tolerant bacteria from olive farms to act as reservoirs of transferable antibiotic resistance, which could be a troublesome issue in clinical settings.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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