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Copper-uptake mediated by an ecofriendly zwitterionic ionic liquid: A new challenge for a cleaner bioeconomy

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ABSTRACT

This study aims to investigate the ability of an imidazolium biobased Zwitterionic Ionic Liquids (ZILs) in enhancing the phytoavailability of copper from garden (G) and vineyard (V) soils using the model plant ryegrass. Uncontaminated and artificially contaminated CuSO₄ soils, unamended and ZIL-amended soil modalities were designed. The copper/ZIL molar ratio (1/4) introduced was rationally established based on molecular modeling and on the maximal copper concentration in artificially contaminated soil. Higher accumulation of copper in the shoots was detected for the uncontaminated and copper contaminated ZIL amended V soils (18.9 and 23.3 mg/kg, respectively) contrary to G soils together with a ZIL concentration of around 3% (W/W) detected by LC-MS analyses. These data evidenced a Cu-accumulation improvement of 38% and 66% compared to non-amended V soils (13.6 and 13.9 mg/kg respectively). ZIL would be mainly present under Cu(II)-ZIL₄ complexes in the shoots. The impact on the chemical composition of shoot was also studied. The results show that depending on the soils modality, the presence of free copper and/or ZIL led to different chemical compositions in lignin and monomeric sugar contents. In the biorefinery context, performances of enzymatic hydrolysis of shoots were also related to the presence of both ZIL and copper under free or complex forms. Ecotoxicity assessment of the vineyard

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soil samples indicated that the quantity of copper and ZIL remaining in the soils had no significant toxicity. ZIL amendment in a copper-contaminated soil was demonstrated as being a promising way to promote the valorization of phytoremediation plants.

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Introduction

Ionic liquids (ILs) are composed of cations and anions and present the significant advantage to possess a structure that can be easily and freely modulated according to the targeted application. They have excellent properties such as good thermal stability, recyclability, wide electrochemical window, low vapor pressure and low flammability (Rodríguez et al., 2020; Seddon et al., 2000; Tian et al., 2021). Some ILs have emerged as “green agents” to replace traditional solvents and have been extensively applied in steel industry (Kim et al., 2018), hydrometallurgy technologies for leaching, recovery, and purification of metal ions (Quijada-Maldolano et al., 2020; Tian et al., 2010). For instance, some ILs have been described to be efficient leaching agents for recovery of copper (Cu) from copper sulfide minerals (Anggara et al., 2019; Hu et al., 2017), brass wastes (Kilicarslan et al., 2014), waste printed circuit boards (Huang et al., 2014). Metals such as Ag and Au have been extracted from electronic wastes by imidazolium-based ILs (Masilela and Ndlovu, 2019). ILs are also good solvents for stabilizing transition metals in catalysis (Crofts et al., 1999). Generally, quaternary ammonium/phosphonium cations or carboxylic acid derived anions-based ILs are the most investigated for heavy metal removal applications (Ramjhan et al., 2021). Hydrophobic ILs and particularly imidazolium-based ILs are often preferred because liquid-liquid extraction is one of the most efficient and simplest technique to separate and concentrate metal ions present in wastewaters (Gonçalves et al., 2021; Inman et al., 2022; Isosaari et al., 2019; Tariq et al., 2021). Meanwhile, hydrophilic imidazolium-based ILs have been intensively studied for their ability to fractionate recalcitrant biopolymers from lignocellulosic biomass in biorefinery processes (Auxenfans et al., 2014; Husson et al., 2018). Because of the questionable ecotoxicity of ILs, zwitterionic ionic liquids (ZILs) have recently emerged as an interesting eco-alternative for lignocellulosic biomass pretreatment with a lower toxicity and a higher biocompatibility with cell membranes (Kuroda et al., 2017; Huet et al., 2020). Among ILs, ZILs are ILs containing negative and positive charge on the same skeleton as zwitterions. Moreover, ZILs can easily form stable complexes with CuCl_2 for recyclable catalysis (Yang et al., 2017).

To the best of our knowledge, no study focused on the ability of ZILs to chelate metals for copper-uptake because some ILs have demonstrated ecotoxicity by the presence of long alkyl chains (Habibul et al., 2021). Natural amino acids have been applied to remove heavy metals but their half-life in the soil generally ranges between 8 and 22 hr for glycine for example which is too short to allow both complexation with metals and Cu-uptake by plants (Henry and

Jefferies, 2003). Ethylenediaminetetraacetate (EDTA) is probably the most efficient chelating agent by increasing the concentration of various metals in above-ground plant tissues (Blaylock et al., 1997; Cunningham et al., 1996; Vassil et al., 1998). Its high efficiency relies on the solubilization of poorly available metals in soils (e.g. Pb/Cr/Cu), followed by a largely passive accumulation of metal complexes in plant shoots through transpiration streams (Elless et al., 2000; Sarret et al., 2001). However, the slow degradation rate and long persistence of EDTA in soil increase the metal leaching risk and make it unsuitable for use under field conditions (Lombi et al., 2001; Meers et al., 2005; Wu et al., 2004). Hence, it is recommended to use biodegradable chelating agents to replace EDTA.

From now on, combining phytoremediation and biorefinery strategies constitutes an additional challenge for a cleaner bioeconomy. Cultivation of dedicated crops in marginal and contaminated lands rather than in fertile and arable lands could avoid conflict with food production while strengthening sustainability of lignocellulosic biorefinery concepts (Gerwin et al., 2018; Hennequin et al., 2022). The main contaminants of agricultural soils in Europe are metals such as copper. When Cu concentration in the soil exceeds 60–125 mg/kg, deleterious effects occur on metabolism and physiology of cultivated agro-resources (Jaswal et al., 2022; Napoli et al., 2019). In term of biorefinery, the biotransformation of these polluted plants is constrained by high concentrations of metals. They negatively impact the activity of hemicellulolytic enzymes used to produce platform sugars (Dhiman et al., 2016; Dhiman et al., 2017; Edgar et al., 2021; Van Assche and Clijsters, 1990). To overcome this, pretreatment of metals-containing plants can be implemented to extract them prior to enzymatic hydrolysis (Hennequin et al., 2022). However, these approaches include numerous steps that can be economically and energetically unprofitable with a questionable sustainability. In this context, we focused on the amendment of a copper-polluted soil with a new imidazolium biobased-ZIL as chelating agent, proven being compatible with hemicellulolytic enzymes and fermentative microorganisms in a previous study (Huet et al., 2020). This present work aims to investigate for the first time the influence of ZIL as chelating agent in copper-polluted soils. The optimal ratio of ZIL:Cu was determined from molecular modeling studies and the corresponding amount of ZIL was introduced into G (Garden) and V (Vineyard) soils. After harvesting the aerial part of the pot-grown of ryegrass (*Lolium perenne* L.), a phytoremediation model plant, the quantification of Cu and ZIL was determined. The impact of the metal-ZIL association on the hemicellulolytic enzymes activity was studied. Ecotoxicity studies of crop soils will be presented and discussed.

1. Materials and methods

In this section, only the non standard procedures used throughout this work are presented. Informations on standard procedures can be found in Appendix A.

1.1. Chemicals and enzymes

Anhydrous sodium acetate (99%) and sodium hydroxide aqueous solution (50%) were purchased from Fluka Sigma-Aldrich (Steinheim, Germany) and acetic acid (99%) from Carl Roth (Lauterbourg, France). Absolute ethanol (> 99.8%) and methanol (> 99.9%), standard L-arabinose, D-galactose, D-glucose and D-xylose were purchased from Sigma-Aldrich (Steinheim, Germany). Glass microfibre filters (< 1 μm pore size) came from Whatman, GE Healthcare Life Sciences (Boston, MA, USA). Cellic CTec2 enzymatic cocktail (specific activity ≥ 1000 FPU/g) including both cellulolytic and hemicellulolytic activities was supplied by Novozymes (Bagsvaerd, Denmark) and prepared at 15 FPU/g of lignocellulosic biomass for the production of hydrolysates rich in monomeric sugars. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 min has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC.

1.2. Computational details

All calculations were performed using the Gaussian09 program (Frisch et al., 2009) at the B3LYP/6-31G* level of theory (Becke, 1988; Becke, 1993; Lee et al., 1988) (see Appendix A for more information).

1.3. Soil sampling and pot experiments

A non-contaminated garden soil (G; clay loam soil) was sampled (0–25 cm) in the north of France (Pont-à-Marcq; 50°31'36"N, 3°06'53"E). In addition, a vineyard soil (V; calcareous cambisol) (Mackie et al., 2012) was sampled (20–40 cm) in the region of Reims (Pévy; 49°19' 0.01"N, 3°49'59.99"E). The sampling depth of the vineyard soil was different to avoid the presence of copper sulfate used by winegrowers. After sampling, soil samples were air-dried, crushed to pass through a 10 mm stainless sieve and homogenized. A randomized pot (9 cm in diameter, 12 cm height; 1 kg of soil) experiment was implemented in a greenhouse for 16 weeks. For both G and V, 24 kg of soil were divided into 8 groups (modalities), triplicates being constituted for each modality. These modalities are summarized in Table 1.

As shown in Table 1, a part of these soils was artificially contaminated by copper sulfate (M5 to M8), the main copper fungicide used by winegrowers to prevent downy mildew of vineyards. The copper concentration of 300 mg/kg in the soil was chosen as it represents the average concentration of Cu in vineyards located in the Reims region (248–519 mg/kg) (Mackie et al., 2012). The amendment was a ZIL derivative described previously (Huet et al., 2020) which was added into the soils in a Cu:ZIL ratio of 1:4 (3.71 g/plant pot) as demonstrated through molecular modeling calculations. The setup

was done in January and pots were placed under controlled conditions in a greenhouse (temperature ranging from 18 to 25°C, light/darkness ratio – 1.4) for 8 weeks. Potted soils were fitted with a bottom cup to prevent leaching, as they were watered with deionized water and maintained once a week at 70% water holding capacity. This was made for all the pots to allow the amendment to have enough contact with Cu and increase the efficiency of complex formation but also to allow the soil microbiota to develop. After 8 weeks, seeds of ryegrass (1 g/pot) were sown. The shoots of ryegrass were harvested 8 weeks after sowing. They were washed twice with deionized water, blotted with filter paper, placed in paper bags and dried in the oven at 40°C until reaching a constant mass. The shoots were weighted before and after drying.

1.4. ZIL extraction from ryegrass shoots

The plant samples were mixed with 50 mL of methanol and after 1 h of stirring at room temperature, the dispersions were filtered. The filtrates containing ZIL derivatives were then diluted to different concentrations to match the standard range. Each solution was filtered through a 0.45 μm membrane for subsequent HPLC-MS analysis (see ESI). ZIL concentrations were determined using a standard range established from pure ZIL derivative.

1.5. Enzymatic hydrolysis of ryegrass shoot samples

Before use, the harvested ryegrass samples were milled with a planetary ball miller (Retsch PM400) for 30 sec at a frequency of 25 sec^{-1} to achieve a size of less than 0.8 mm then freeze-dried (water content of 0.8% \pm 0.1% W/W). The enzymatic hydrolysis procedure is adapted from our previous study (Araya-Farias et al., 2019) (Appendix A).

1.6. Sugar composition of ryegrass shoot samples and quantification of water-soluble sugars

The sugar composition of ryegrass and the solubilized sugars content in hydrolysates were determined by HPAEC-PAD according to previous studies (Husson et al., 2018; Rémond et al., 2010) (see Appendix A).

1.7. Klason lignin analysis

Relative lignin content was determined from the standard NREL method (Shill et al., 2011; Sluiter et al., 2012) (see Appendix A).

1.8. Ecotoxicity assessment

The toxicity towards the photobacterium *Aliivibrio fischeri* was measured using the BioTox™ LumoPlate™ Ultimate Matrix kit (EBPI, Mississauga, Ontario, Canada), a commercial bioassay widely used in ecotoxicological research and risk assessment of either water samples or sediment and soil samples. Briefly, the sample toxicity was evaluated using a Varioskan™ LUX multimode microplate reader (ThermoScientific, Fisher Scientific SAS, Illkirch, France) by quantifying the inhibition of bioluminescence emitted by the bacteria after a contact time

Table 1 – Modalities of the experimental design.

Modality	Soil	Description
M1	G/V	uncontaminated soil, unamended and without ryegrass
M2	G/V	uncontaminated soil, unamended and with ryegrass
M3	G/V	uncontaminated soil, amended with ZIL and without ryegrass
M4	G/V	uncontaminated soil, amended with ZIL and with ryegrass
M5	G/V	artificially contaminated soil, unamended and without ryegrass
M6	G/V	artificially contaminated soil, unamended and with ryegrass
M7	G/V	artificially contaminated soil, amended with ZIL and without ryegrass
M8	G/V	artificially contaminated soil, amended with ZIL and with ryegrass

of 30 min. For each of the 8 vineyard soil samples (all samples were led in triplicate), 2 g of soil are mixed in 8 mL of sample diluent for 5 min (LumoPlate™ Ultimate Matrix kit, reagent 1243–125), and then a serial dilution by half of each sample provides concentration-dependent differences in inhibitory effects used to calculate EC₅₀ (effective concentration causing 50% inhibition of light output). An increasing toxicity causes a decrease in bacterial luminescence.

1.9. Statistical analysis

Statistical analyses were performed using the XLSTAT 2019 software (Addinsoft, Paris, France). The Shapiro–Wilk test was used to test the normality distribution of the data. ANOVA and Tukey's tests were conducted in order to identify significant differences between individual treatments. Differences were considered as statistically significant at $p < 0.05$.

2. Results and discussion

2.1. Ecotoxicity

As a prerequisite of the use of any compounds for depollution, ecotoxicity of 3-(1,3-diethyl-1H-imidazol-3-ium-4-yl)propanoate (ZIL, see ESI for structure) was evaluated using the *Aliivibrio fischeri* test (BioTox™ LumoPlate™ Ultimate Matrix kit). While, as expected, CuSO₄ had a huge toxicity (EC₅₀ = 1.8 mg/L), obtained EC₅₀ of ZIL alone was around 200 mg/mL, indicating the low toxicity of these compounds. The EC₅₀ in the bioluminescence of *Aliivibrio fischeri* was then determined using 7 dilutions of each soil samples. After 30 min of exposure, for all the vineyard soil samples, independently of the treatment, EC₅₀ was between 12.48 and 13.58 mg/mL indicating a much higher toxicity than ZIL compound. The ecotoxicity data also showed that Cu contamination, amendment with ZIL and the presence of ryegrass had no significant effect on the soil toxicity (Appendix A Table S1).

Few works have already studied such ionic compounds and a close structure-toxicity dependence has been reported (Abbas et al., 2018; Rantamäli et al., 2017). The present results let us suggest the presence of other contaminants in the soil with higher toxicities than copper such as organic contaminants as polychlorobiphenyls (PCB), polycyclic aromatic hydrocarbons (PAH) and/or compounds used for their pesticide activity (Fernández-Alba et al., 2002; Tóth et al., 2019; Jarque et al., 2016). These results demonstrated the eco-

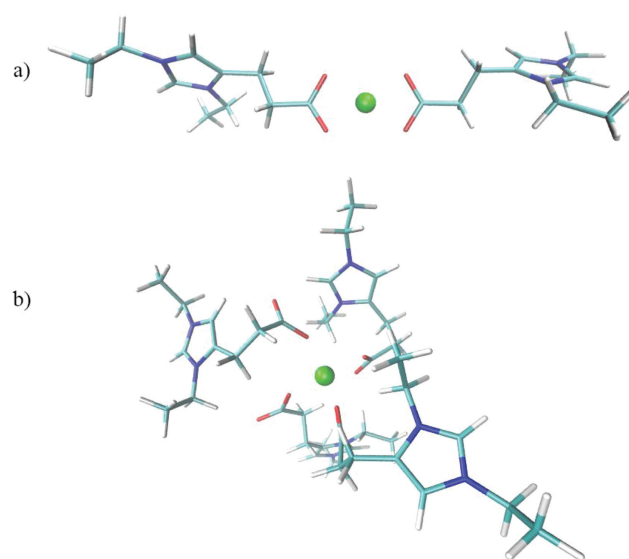


Fig. 1 – Chemical structures of the Cu(II)-(ZIL)₂ (a) and Cu(II)-(ZIL)₄ complexes (b).

compatibility of ZIL and justified its use as potential chelating agent for copper phytoremediation study from different soils.

2.2. Computational modeling

Firstly, molecular modeling was performed to set the ZIL quantities necessary to complex copper. It is well established that the most stable Cu(II) complexes are four-coordinated structures (Pavelka and Burda, 2005). To establish four bonds between ZIL (Fig. 1) and Cu(II), many Cu(II)-ZIL complexes have to be considered as ZIL can bind Cu(II) in a monodentate or bidentate way. Hence, here we present the Cu(II)-(ZIL)_n ($n = 2-4$) complexes where Cu(II) interacts in a four-coordinated way with its ligands. Different geometries of the complexes were built and their binding free energies were evaluated and compared (Appendix A Table S2).

In the model and simplest Cu(II)-ZIL complex, ZIL is bound to Cu(II) in a bidentate way; the cation-oxygen distances are equal to 1.87 Å. The interaction energy between Cu(II) and ZIL is of -318 kcal/mol. Adding a second ZIL to Cu(II) forms a very stable Cu(II)-(ZIL)₂ complex as the coordination shell of Cu(II) is now complete. Compared to the Cu(II)-ZIL complex, the system is more stable, its binding energy is -462 kcal/mol, the four

Cu(II)-oxygen distances are equal and a bit longer than in the Cu(II)-ZIL complex. The interaction between the two ZILs is negligible in the complex as they are 180° apart. Hence, the Cu(II)-ZIL bidentate interaction accounts for ~ -231 kcal/mol in a four coordinated Cu(II) complex.

Because of steric hindrance, the three ZILs in the Cu(II)-(ZIL)₃ complex are unable to interact with Cu(II) in a bidentate way. In order to accommodate the three ligands and being four-coordinated, Cu(II) interacts with one ZIL in a bidentate way (the Cu(II)-oxygen distances are longer than in the Cu(II)-(ZIL)₂ complex) and with the two others in a monodentate way. Upon addition of a third ZIL, the interaction energy in this system is lowered compared to the two previous complexes and is of -506 kcal/mol. The addition of a fourth ZIL around Cu(II) results either in four monodentate interactions with Cu(II) directly surrounded by the four ZILs or in two bidentate interactions, with two ZILs in a second shell, the latter being less favored (results not shown). Again, adding another ligand around Cu(II) results in a lower interaction energy, the latter being of -523 kcal/mol between Cu(II) and four ZILs bound in a monodentate way, allowing the estimation of the Cu(II)-ZIL monodentate interaction, which accounts for ~ -131 kcal/mol. Using a simple additive model, the estimated binding energy in the Cu(II)-(ZIL)₃ complex should be -493 kcal/mol, which is very close to the -506 kcal/mol obtained by the calculation, thus validating an energy contribution for the two kinds of Cu(II)-ZIL interactions.

These results show and confirm that Cu(II) is stable when coordinated to four atoms with an optimal Cu(II)-O distance of ~1.95 Å. On the first hand, Cu(II)-(ZIL)₂ is more stable than Cu(II)-(ZIL) and on the other hand, upon addition of ZIL molecules, the system rearranges itself to interact with four oxygen atoms. The Cu(II)-(ZIL)₂ and Cu(II)-(ZIL)₄ complexes (Fig. 1) both feature four copper-oxygen distances in the 1.95 Å range (Appendix A Table S3), hence showing the strongest bonds. Moreover, the binding energy of the Cu(II)-(ZIL)₄ being the smallest despite potential steric hindrance, this complex is the most stable and its formation seems the most probable. Therefore, it was decided to use a ZIL:Cu ratio of 4:1 to form complexes for the experimental part of this work. To support the different Cu(II)-ZIL complexes, mass spectrometry analysis were performed (Appendix A Fig. S2). Cu(II)-ZIL, Cu(II)-(ZIL)₂, Cu(II)-(ZIL)₃ and Cu(II)-(ZIL)₄ complexes were detected and are in good agreement with each theoretical isotope model.

2.3. Effect of ZIL and ryegrass on pH, CaCO₃ and bioavailability of Cu

Before potting, pH, CaCO₃ and the total Cu concentrations of the two alkaline soils were measured. Procedures for soil, plant analyses and extractable Cu are described in Appendix A. The results are summarized in Appendix A Table S4. However, the average total carbonate content of the V soil is approximately 14-fold higher than that in the G soil (152.7 g/kg vs 10.6 g/kg) leading to higher alkaline pH (8.2 vs 7.4). These results correlate well with the pedological characteristic of vineyard soils in the Reims Region, described as calcaric cambisol (Mackie et al., 2012). The average concentration of Cu in G soils (15.5 mg/kg) is of the same order of magnitude as those in

the north of France (Bellabio et al., 2018; Panagos et al., 2018) whereas in V soils this concentration (33.5 mg/kg) is from 3 to 10 times lower than those reported for french vineyard soils (Mackie et al., 2012; Panagos et al., 2018) This result is not surprising since it is well known that the main mechanism of Cu retention in carbonated soil is the surface precipitation of CuCO₃ and malachite (Cu₂(OH)₂CO₃) or Cu-Ca substitution in calcite (Ma et al., 2006; Ponizovski et al., 2007). This could explain why the extractable Cu concentrations using water and NH₄NO₃ were very low (from 1.3% to 2.8%) in V soil. The percentage of extractable Cu in G soil is also very low (ranging from 1.6% to 5.0%). This observation could be related to the slightly alkaline pH (7.4), in the 7–8 range in which solubility of both anionic and cationic forms of Cu decreases. Indeed, Cu concentration in uncontaminated soil is lower than 1 mg/kg (Ponizovski et al., 2007; Sauvé et al., 1997).

After the pot experiments, pH, CaCO₃ and Cu concentrations in water and NH₄NO₃ extracts were measured for each modality (Table 2). As shown in Table 2, significant differences were obtained between data from G and V soils. Despite the slight acidic properties of copper sulfate and the slight alkaline properties of ZIL, both artificial contamination with Cu and/or amendment with ZIL had no effects on pH values and CaCO₃ concentrations in absence or presence of ryegrass. The Cu-extractable concentrations in artificially contaminated G and V soils were significantly higher than those in non-contaminated soils. It is worth noting that the water Cu-extractable concentrations tend to significantly increase under ryegrass cultivation with or without ZIL (G-M8, V-M6 and V-M8). Clearer results were obtained using nitrate ammonium, a solvent widely used to assess the bioavailability of Cu (Wang et al., 2017). Once again, the extractable Cu concentrations were the highest in amended G soils after ryegrass production (G-M8). The most interesting results were obtained using the vineyard soil (Table 2). Considering the non-artificially contaminated V soil, the highest Cu-extractable concentrations were measured for modalities V-M2 and V-M4. Compared to the results obtained with V-M1 and V-M3, no statistical effect of the system “ryegrass + ZIL” on the bioavailable concentrations of Cu can be reported. In artificially contaminated V soils, surprising antagonistic results were highlighted for V-M6 and V-M8. Compared to V-M5, it was clearly demonstrated that ryegrass significantly increases the NH₄NO₃-extractable concentration of Cu in this V soil (from 3.94 to 7.38 mg/kg for V-M5 and V-M6, respectively) meaning that the speciation of Cu in the artificially contaminated V soil is different than that in the other one. The alkaline pH and the total carbonate content of the V soil suggest the presence of copper as Cu(OH)₃⁻, Cu(OH)₄²⁻ and Cu(CO₃)₂²⁻ as well as Cu-organic complexes like those between Cu and soluble organic matter (SOM) and/or dissolved organic matter (DOM) (Kabata-Pendias et al., 2004). Unexpectedly, the NH₄NO₃-extractable concentrations of Cu significantly decrease after ryegrass production on the V soil amended with ZIL. This result suggests the formation of (i) a non-extractable complex between Cu and ZIL, (ii) a complex able to promoting the transfer of Cu(II) in ryegrass and/or (iii) a complex uptake and transport by ryegrass. Recently, Habibul et al. (2019) demonstrated the ability of ryegrass to accumulate and to metabolize ILs (1-butyl-3-methylimidazolium bromide) from water. The authors

Table 2 – pH, CaCO₃ and extractable Cu concentrations before and after pot experiments and depending on treatments.

Soil/modality	pH	CaCO ₃ (g/kg)	Water extractable [Cu] (mg/kg)	NH ₄ NO ₃ extractable [Cu] (mg/kg)
G-M1	7.4 ± 0.1 ^a	10.5 ± 0.3	0.8 ± 0.00 ^a	0.26 ± 0.00 ^a
G-M2	7.5 ± 0.1 ^a	10.1 ± 0.4	0.84 ± 0.02 ^a	0.29 ± 0.01 ^a
G-M3	7.5 ± 0.1 ^a	10.2 ± 0.4	0.77 ± 0.01 ^a	0.25 ± 0.00 ^a
G-M4	7.9 ± 0.0 ^a	10.4 ± 0.3	0.82 ± 0.00 ^a	0.29 ± 0.01 ^a
G-M5	7.3 ± 0.1 ^a	10.7 ± 0.3	1.33 ± 0.03 ^b	1.31 ± 0.01 ^b
G-M6	7.6 ± 0.0 ^a	10.8 ± 0.8	1.36 ± 0.14 ^b	1.37 ± 0.08 ^b
G-M7	7.4 ± 0.1 ^a	12.9 ± 0.5	1.44 ± 0.10 ^b	1.37 ± 0.05 ^b
G-M8	7.6 ± 0.1 ^a	11.7 ± 1.9	1.60 ± 0.01 ^c	1.56 ± 0.15 ^c
V-M1	8.1 ± 0.1 ^A	153.5 ± 7.4	0.97 ± 0.02 ^A	0.43 ± 0.01 ^A
V-M2	8.1 ± 0.1 ^A	166.3 ± 5.5	1.00 ± 0.03 ^A	0.56 ± 0.01 ^B
V-M3	8.1 ± 0.0 ^A	154.5 ± 10.3	0.93 ± 0.01 ^A	0.44 ± 0.03 ^A
V-M4	8.2 ± 0.0 ^A	180.3 ± 5.2	1.03 ± 0.01 ^A	0.60 ± 0.01 ^B
V-M5	8.0 ± 0.0 ^A	148.7 ± 11.9	1.42 ± 0.02 ^B	3.94 ± 0.01 ^C
V-M6	8.2 ± 0.0 ^A	158.5 ± 5.4	1.79 ± 0.09 ^C	7.38 ± 0.05 ^D
V-M7	8.1 ± 0.1 ^A	177.4 ± 13.8	1.48 ± 0.04 ^B	3.93 ± 0.77 ^C
V-M8	8.2 ± 0.1 ^A	158.9 ± 3.4	1.93 ± 0.20 ^C	1.43 ± 0.20 ^E

Letters a, b, c, A, B, C, D, E denote significant differences ($p < 0.05$).

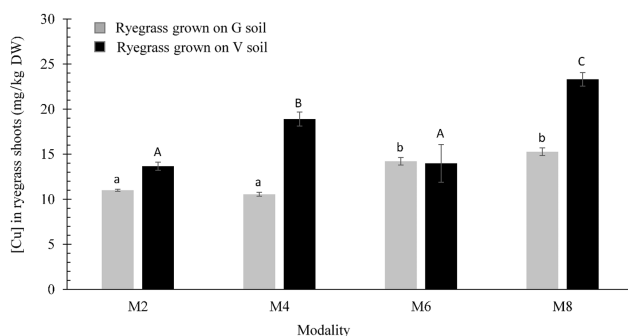


Fig. 2 – Cu concentrations in ryegrass shoots on uncontaminated soil (M2), artificially contaminated soil (M6), uncontaminated soil with ZIL (M4) and artificially contaminated soil with ZIL (M8). Letters a, b, c, and A, B, C denote significant differences between concentrations of Cu in ryegrass from G and V soils, respectively ($p < 0.05$).

showed the presence of this IL in the ryegrass shoots, highlighted its translocation to the shoots and revealed that the concentration of IL in roots was higher than in shoots. In view of the poor knowledge on this subject, we decided to complete this study by measuring the concentration of copper and ZIL in the shoots of ryegrass.

2.4. Concentration of Cu and ZIL in ryegrass shoots

Concentrations of Cu were measured in the shoots of ryegrass grown on G and V soils according to the M2, M4, M6 and M8 modalities. As reported in Fig. 2, the concentration of Cu in ryegrass shoots grown on artificially contaminated G soils (M6/M8) were significantly higher than those grown on the uncontaminated G soils (M2/M4). No significant effect of the ZIL amendment was reported on the translocation of Cu from the rhizosphere soil to the aboveground part of ryegrass. Results obtained from ryegrass grown on the vineyard soil were much more interesting. As shown in Fig. 2, the concentrations of Cu

in the shoots of ryegrass grown on V-M2 and V-M6 modalities were statistically the same (13.7 ± 0.5 mg/kg and 14.0 ± 2.1 mg/kg, respectively). Like all micronutrients, Cu is essential for plant growth and regulation of Cu uptake by plants is necessary to avoid toxicity effects. For ryegrass, the herbage concentration of Cu is between 2 and 15 mg/kg DW and above 20 mg/kg DW, phytotoxicity effects of Cu were reported in ryegrass (Healy et al., 2016). Interestingly, the concentration of Cu in the shoots of ryegrass grown on amended V soils by ZIL (M4/M8) significantly increased compared to the unamended conditions (M2/M6) and were 18.9 ± 0.8 mg/kg DW and 23.3 ± 0.8 mg/kg DW. Neither visual symptom of Cu phytotoxicity in shoots of ryegrass, nor negative effect of ZIL and/or Cu concentration on the fresh biomass of ryegrass shoots were observed. These results correlated well with the study of Huet et al. (2020) in which the biocompatibility of ZIL with microorganisms was reported. On the other hand, Habibul et al. demonstrated the ability of ryegrass to uptake ILs (1-butyl-3-methylimidazolium bromide) (Habibul et al., 2019).

The presence of ZIL, used as amendment in the current study, drastically increased the Cu uptake by ryegrass (from 38% in V-M4 vs V-M2 and 66% in V-M8 vs V-M6) suggesting that ZIL promotes free copper ions in the rhizosphere and/or the complex “Cu(II)-ZIL” is highly phytoavailable.

Next, to determine ZIL concentrations in ryegrass shoots and to simplify the analyses, we decided to examine data only on V samples as the most interesting results on Cu uptake. Based on recent publications demonstrating that imidazolium-based ILs can be absorbed by plants (Chen et al., 2018; Xu et al., 2018; Liu et al., 2018a, Liu et al., 2018b; Hu et al., 2020; Habibul et al., 2021), we undertook to extract ZIL from the different ryegrass samples. First, the dried ryegrass shoots were extracted with MeOH then injected and analyzed by LC-MS (Appendix A Figs. S3–S5). ZIL accumulation in shoots was expressed as the ratio of ZIL mass to dried ryegrass shoots (W/W). As expected, ZIL derivatives were not detected in the uncontaminated and unamended samples (V-M2 and V-M6). Concerning the ZIL-amended modalities, the V-M4 sample

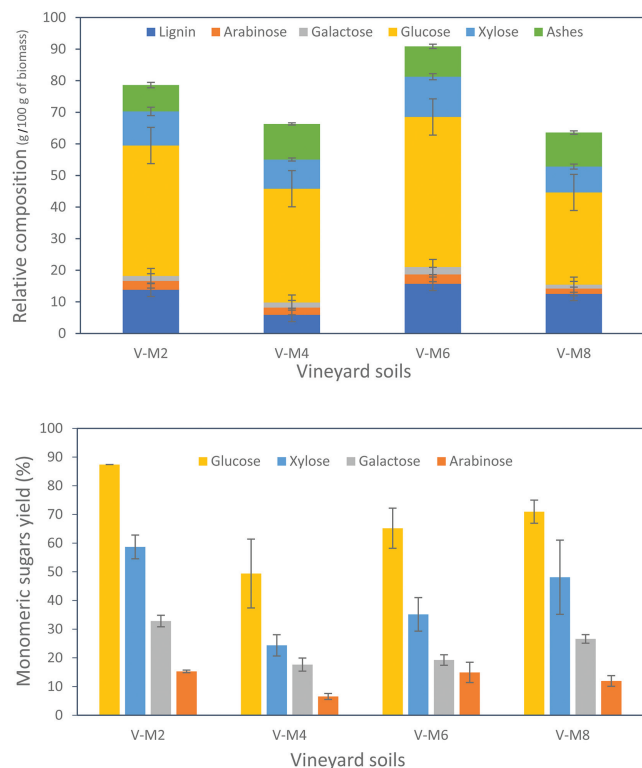


Fig. 3 – Top: relative composition (g/100 g of dry matter) in monomeric sugars and Klason lignin of aerial part from ryegrass cultivated on the different soils. Bottom: monomeric sugar yields (%) achieved after 72 hr of enzymatic hydrolysis of the aerial part from ryegrass (2% W/V). Hydrolysis were performed in triplicate and catalyzed by Cellic CTec2 cocktail (15 FPU/g of ryegrass). V-M2: uncontaminated soil, V-M4: uncontaminated soil with ZIL, V-M6: artificially contaminated soil and V-M8: artificially contaminated soil with ZIL.

contains similar concentration of ZIL ($3.47\% \pm 0.13\%$) compared to the V-M8 sample ($3.26\% \pm 0.07\%$). These very low percentages indicate that the majority of ZIL remains in the soil and/or has been metabolized by ryegrass plants (Habibul et al., 2021).

2.5. Impact of Cu-concentration on chemical composition of ryegrass

Plant chemical composition depends on many factors particularly growth conditions. As micronutrient, a minimal amount of copper is needed to ensure cellular functions but an excess may induce detrimental effects on cell wall synthesis (Printz et al., 2016). Therefore, chemical composition of harvested ryegrass was determined for the different vineyard soils: uncontaminated soil with or without ZIL (V-M2/V-M4) and artificially contaminated soil with or without ZIL (V-M6/V-M8). The compositions in monomeric sugars and Klason lignin are presented in Fig. 3 (top). It is important to stress that the copper/ZIL molar ratio (1/4) was fixed on the basis of maximal copper concentration in V-M6 and V-M8 soils leading to similar ZIL amounts in V-M4 and V-M8.

V-M2 soils used to grow ryegrass exhibited a natural concentration in copper leading to a copper accumulation of 13.7 mg/kg in ryegrass shoots. The chemical composition of this harvested biomass is consistent in term of monomeric sugars and Klason lignin contents with literature data (Sun et al., 2021). After ZIL amendment in soil (V-M4), an increase of 38% of copper accumulation in shoots (18.7 mg/kg) was observed as well as an accumulated ZIL concentration of 3.47% W/W confirming the ability of ZIL to improve the phytoavailability of Cu. A significant decrease of 67% in relative Klason lignin content and no modification of sugar amounts were observed in comparison with V-M2. This decrease in lignin content could be explained by a lower bioavailable copper concentration in V-M4 compared to V-M2 due to the large excess of ZIL probably affecting plant metabolism (Ślusarczyk et al., 1986). Indeed, the copper in this modality was suggested to be in the Cu(II)-ZIL complex form making it unavailable as essential cofactor for the oxydoreductases implied in lignin biosynthesis (Chen et al., 2002; Liu et al., 2018a, Liu et al., 2018b).

The shoots harvested from artificially contaminated soil (V-M6) exhibited a similar copper concentration (14.0 mg/kg) than those obtained with uncontaminated soil (V-M2), demonstrating the requirement of chelating agent addition to hyper phytoremediation. About sugars and Klason lignin contents, no significant change of composition was observed contrary to what can be evidenced with other more toxic heavy metals (Waliszewska et al., 2019).

As expected, when ZIL amendment was performed on artificially copper contaminated soil (V-M8), a higher accumulation of copper in the shoots was detected (23.3 mg/kg) together with a similar ZIL concentration of 3.26% W/W. These data resulted in a translocation improvement of 66% compared to V-M6. The Klason lignin content of ryegrass grown on V-M8 reached a similar value to the one obtained using V-M6 modality whereas glucose content decreased (29.2 g/100 g vs 47.5 g/100 g). ZIL was supposed to be mainly present under the Cu(II)-ZIL complex form. Since the copper concentration is higher, a significant part would thus remain bioavailable to ensure oxydoreductases-catalyzed biosynthesis of lignin while affecting sugar metabolism of ryegrass (Ślusarczyk and Ruszkowska, 1986; Chen et al., 2002; Liu et al., 2018a, Liu et al., 2018b).

2.6. Impact of Cu-concentration on enzymatic hydrolysis of harvested biomass

A purpose of this work is to investigate the potential in terms of biorefinery of ryegrass as a phytoremediation plant. For this reason, enzymatic hydrolysis of polysaccharide fractions from harvested biomass was studied according to the copper and ZIL accumulation in shoots depending on the V-soil modalities used. For the implementation of these hydrolyses, the commercial CellicCTec2 cocktail was selected as it contains (hemi)cellulolytic activities allowing to target both cellulose and hemicellulose fractions (Araya-Farias et al., 2019). The monomeric sugar yields (%) achieved after 72 hr of enzymatic hydrolysis are presented in Fig. 3 (bottom).

Whatever the modalities, the two major monosaccharides released after the enzymatic hydrolysis of ryegrass shoots were glucose and xylose in agreement with the initial chemi-

cal composition. Concerning the ryegrass cultivated on V-M2, enzymatic hydrolysis led to glucose and xylose with yields of 87.4% and 58.7%, respectively. Contrary to the yields reported in the literature and obtained from ryegrass with other enzymatic cocktails (Sun et al., 2021; Martín et al., 2008), Cellic CTec2 preparation would be particularly suitable for an efficient bioconversion of this biomass into fermentable or platform sugars. The recalcitrance of this biomass would not thus require a pretreatment step for allowing the accessibility of these enzymes to polysaccharide fractions. We also demonstrated that a copper concentration of 13.7 mg/kg in ryegrass shoots (V-M2) would not induce negative effects on hemicellulolytic activities.

Enzymatic hydrolysis of ryegrass cultivated on V-M4 provided the lowest glucose and xylose yields. Although we previously demonstrated that ZIL amendment led to significantly decrease the Klason lignin content, this lesser degree of lignification did not improve enzymatic digestibility of polysaccharide fractions. As the amount of ZIL was calculated on the basis of artificially contaminated soils, in other words at a maximum copper concentration, ZIL should be in large excess under these conditions. Thus, we suggested that a significative amount of free ZIL induced partial deactivation of hemicellulolytic enzymes.

Concerning the ryegrass cultivated on V-M6, performances of enzymatic hydrolysis were slightly decreased as illustrated by respective glucose and xylose yields of 65.2% and 35.2%. As the accumulated copper concentration in shoots was similar to the one of V-M2, we assumed that the V-M6 modality (artificially contaminated soil) impacted negatively the enzymatic digestibility of polysaccharide fractions from the harvested biomass (Dhiman et al., 2017).

Compared to V-M4, the presence of ZIL in V-M8 did not seem to generate the same trend. The glucose and xylose yields were superior (71.0% and 48.1%, respectively) with a higher copper accumulation (23.3 mg/kg) in shoots. Indeed, in V-M8 modality, the amount of ZIL introduced is such that all copper should be complexed. Only a small amount of free ZIL should be present, thus inducing a weak deactivation of hemicellulolytic enzymes allowing competitive hydrolysis performances. To support this hypothesis, enzymatic hydrolysis of ryegrass cultivated on V-M8 was also accomplished by introducing additional free ZIL (3.26 g per 100 g of ryegrass, corresponding to the accumulated ZIL concentration detected in shoots). The achieved yields in monomeric sugar were significantly decreased in these hydrolysis conditions (Appendix A Fig. S6). This strengthens our hypothesis about hemicellulolytic enzymes deactivation induced by the free ZIL. Our results suggested that the ZIL amendment in a copper-contaminated soil would represent a promising way to promote the valorization of phytoremediation plants.

3. Conclusions

We have taken advantage of ZIL ability to complex metal ions and their compatibility with (hemicellulolytic) enzymes to efficiently combine phytoremediation and biorefinery on soil. From molecular modeling study, it appears that copper com-

plexes featuring 4 ZILs are favored, which allowed us to set the amount of ZIL to use. The ryegrass used here as a phytoremediation model plant was grown on two representative soils (G and V), one of which is richer in CaCO₃. To these two soils were artificially added CuSO₄ and/or ZIL compounds. Copper analyses show that the best results for Cu uptake was observed on V soils with a concentration increase of 38% (18.9 mg/kg), when the soil has been amended with ZIL (V-M2 vs V-M4) and of 66% (23.3 mg/kg) when the soil has been artificially contaminated with copper and ZIL derivatives (V-M6 vs V-M8). The combination of ZIL and high CaCO₃ in V soils presents the highest Cu extraction demonstrating that ZIL promotes the transfer of Cu in ryegrass shoots. These results are particularly relevant for application on vineyard soils polluted with amendments such as Bordeaux mixture.

Depending on the soil modality (contamination level on copper), the presence of “free copper”, “free ZIL” or “Cu-ZIL complexes” led to different chemical compositions in lignin and monomeric sugars contents. Similar trends were also observed in the enzymatic hydrolyses.

The high EC₅₀ value of ZIL, 200.24 ± 27.38 mg/mL demonstrates the non-toxicity of this compound. The quantity of copper and ZIL introduced in this study has no ecotoxicity compared to control soil.

Declaration of Competing Interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this article.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.10.011.

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