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Effect of *Pseudomonas putida*-producing pyoverdine on copper uptake by *Helianthus annuus* cultivated on vineyard soils

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Abstract

Bioaugmentation-assisted phytoextraction was used to reduce the Cu load in vineyard soils.

While performance is usually the end point of such studies, here we identified some mechanisms underlying Cu soil to plant transfer, particularly the role of siderophores in the extraction of Cu from the soil-bearing phases and its phytoavailability.

Carbonated vs. non-carbonated vineyard soils were cultivated with sunflower in rhizoboxes bioaugmented with *Pseudomonas putida*. *gfp*-tagged *P. putida* was monitored in the soil and pyoverdine (Pvd), Cu, Fe, Mn, and Zn were measured in the soil solution. Trace elements (TE) were analysed in the roots and shoots. Plant growth and nutritional status were also measured.

With bioaugmentation, the concentration of total Cu (vs. Cu^{2+}) in the soil solution increased (decreased) by a factor of 1.6 to 2.6 (7 to 13) depending on the soil. The almost 1:1 relationship between the excess of Fe+Cu mobilized from the solid phase and the amount of Pvd in the soil solution in bioaugmented treatments suggests that Pvd mobilized Fe and Cu mainly by ligand-controlled dissolution via a 1:1 metal-Pvd complex. Bioaugmentation increased the Cu concentration by 17% in the shoots and by 93% in the roots, and by 30% to 60% the sunflower shoot biomass leading to an increase in the amount of Cu phytoextracted by up to 87%. The amount of Fe, Mn, Zn, and P also increased in the roots and shoots. Contrary to what was expected, carbonated soil did not increase the mobilization of TE. Our results showed that bioaugmentation increased phytoextraction, and its performance can be further improved by promoting the dissociation of Pvd-Cu complex in the solution at the soil-root interface.

Keywords: Bacterial survival; Bioaugmentation; Iron availability; Phytoextraction; Siderophore

1. Introduction

Vineyards represent 3% of French arable land, which corresponds to 20% of the phytosanitary products marketed (Aubertot et al. 2005). With the presence of contaminants in wine-growing soils resulting from vine treatments, the sustainability and durability of these agrosystems is called into question and a search is underway for other possible practices to ensure they are part of an agro-ecological approach. Among the contaminants, copper (Cu), which has been used for more than a century to control downy mildew, is a threat to the long-term sustainability of these wine-growing ecosystems.

Copper is a trace metal naturally occurring in soils at concentrations usually ranging from 13 to 24 mg kg⁻¹ (Kabata-Pendias 2001). For example, 90% of French soils naturally contain between 5 and 36 mg Cu kg⁻¹, with an average of 20 mg kg⁻¹ (GIS Sol 2011). However, depending on the frequency of Cu-fungicide treatments, over concentrations have been measured, which vary greatly from one region to another and even within a wine growing terroir. A meta-analysis conducted by Ballabio et al. (2018) at the European scale showed that 14.6% of the vineyard soils sampled showed concentrations of Cu higher than 100 mg kg⁻¹. According to a recent meta-analysis (Karimi et al. 2021), the maximum annual copper dose authorised by the European Commission in 2018 (max. 4 kg kg⁻¹ year⁻¹ on average over a period of 7 years) should not substantially modify the quality or biological functions of the soil. However, the cumulative effect of copper inputs over decades at annual doses much higher than those currently in use may affect soil life, as shown in several studies devoted to the microbial component. In the study of Lejon et al. (2008) with soil microcosms from long-term field experiment contaminated at 250 mg Cu kg⁻¹, the negative effect of Cu on microbial biomass and diversity was shown to be higher in the absence of organic matter added to the soil, and even higher when Cu was recently added. Regarding earthworms, a harmful effect on their abundance and activity has been reported at Cu concentrations starting at around 30 mg kg⁻¹ (Eijsackers et al. 2005; Paoletti et al. 1998). Regulations in Australia, New Zealand and Slovenia recommend an environmental impact study starting from 60 mg Cu kg⁻¹ soil (Pietrzak and Mc Phail 2004; Rusjan et al. 2007). Unlike conventional winegrowers, no alternative products to Cu for mildew control are available to organic winegrowers, resulting in additional enrichment of soils with Cu despite the reduction in doses voted by EC (European Commission)-

Phytoextraction is a potentially relevant way to limit the Cu enrichment of soils, and even to compensate for inputs. If applied in the inter-row of vineyards or to fallow vineyard soils,

phytoextraction would not only reduce the copper load but also the risk of erosion. It is also conceivable that copper can be recycled from biomass. Unfortunately, no terrestrial Cu-hyperaccumulating plants have been identified to date. Phytoextraction experiments are often performed with plants with Cu concentrations that usually do not exceed 100 mg kg⁻¹ of shoots (for reviews, see Lange et al. 2017; van der Ent et al. 2013), which means that the amount of copper that can be expected to be recovered would at best reach 1 kg⁻¹ ha⁻¹, based on a biomass production of 10 tons ha⁻¹. Among other factors, this moderate accumulation of Cu can be explained by its low phytoavailability in the soil, which decreases as early as seven days after vineyard treatment (Lejon et al. 2007, 2008). Indeed, Cu exhibits a strong affinity for solid phases, especially solid organic matter (Degryse et al. 2009; Manceau and Matynia 2010), and its concentration in pore water is usually low, i.e., <1 µM even in Cu-contaminated vineyard soils (Ferreira et al. 2018; Soja et al. 2018). The form of copper in solution also determines its phytoavailability, Cu²⁺ being the main chemical form removed by plants according to the most commonly used free-ion-activity model (FIAM) (Campbell, 1995). However, free copper activity is low in natural soil solutions because many organic and inorganic ligands interact with copper. In particular, dissolved organic matter has a very high affinity for copper (Arias et al. 2004). Different mechanisms can be used to increase the concentration of Cu in the soil solution, in the hope that it is in a phytoavailable chemical form, as reviewed by Cornu et al. (2017b, 2021). Some soil bacteria (e.g., *Pseudomonas*, *Streptomyces*) release complexing molecules including siderophores to meet their Fe requirements and are thus categorised as siderophore-producing bacteria (SPB). Siderophores are low molecular weight organic chelators (150-2000 Da), characterised by a very high affinity for Fe(III) (>10³⁰ M⁻¹). Siderophores can also chelate divalent transition cations (Braud et al. 2009). Pyoverdine (Pvd) from *P. aeruginosa* has a non-negligible binding affinity for Cu of 10^{20.1} M⁻¹ (Cornu et al. 2014). Using a

collection of vineyard soils at different pH (5.9 to 8.6), Cornu et al. (2019) demonstrated that the mobility of Cu, estimated via total Cu in soil pore water, increased by a factor of between 1.4 and 8 with a supply of 200 $\mu\text{mol kg}^{-1}$ soil of purified Pvd from *Pseudomonas fluorescens*, making this bacterial species a good candidate to improve phytoextraction performance. Previously Cornu et al. (2014) showed that Pvd not only increased the mobility but also the phytoavailability and the uptake of Cu by tomato and barley. As reported for various metals, including Cu (Dimkpa 2016; Lebeau et al. 2008; Rajkumar et al. 2010; Sessitsch et al. 2013), SPB can enhance Cu phytoavailability by altering Cu dynamics at the soil-root interface and, like promoting growth plant rhizobacteria (PGPR), are able to stimulate plant growth. Bacteria for bioaugmentation-assisted phytoextraction are generally selected for their potential to produce compounds including siderophores, indole acetic acid, 1-aminocyclopropane-1-carboxylate deaminase. But only plant removal of trace elements (TE) is measured, while the complete process chain has only rarely been studied. Thus, the high variability of the effect of inoculation on phytoextraction yield is not well explained. The aim of the present study was to evaluate the role of *Pseudomonas putida* previously selected from vineyard sediments (Braud et al. 2015) on the phytoavailability and rate of phytoextraction of Cu and other TE in a carbonated and non-carbonated vineyard soils with respectively low and high concentrations of mobile iron. Indeed, Pvd production is known to be stimulated when the concentration of mobile iron is below 10 μM up to 50 μM (de Villegas et al. 2002; Manninen and Sandholm 1993; Sayyed et al. 2005). This should lead to larger amounts of Cu being extracted from carbonated soils. Sunflower plants were cultivated in rhizoboxes to investigate the mechanisms involved at the soil-root interface in depth. Soil colonisation by *gfp*-tagged *P. putida* was measured along with Pvd, Cu (by distinguishing

Cu²⁺ and complexed Cu) and other TE recovered in the soil solution. The effect of soil bioaugmentation on phytoextraction performance and plant fitness was also measured.

2. Material and methods

2.1. Soils

Two sandy soils developed on gravel (one non-carbonated (NC) with < 1 g kg⁻¹ and the other carbonated (C) with 9 g kg⁻¹) were collected in a Bordeaux vineyard (Graves, Pessac-Leognan area, France) in the 0-30 cm soil layer, which corresponds to the tilled soil horizon. The CEC of both soils is low, and they are both poor in organic matter. Aside from the CaCO₃ content, the main difference between the soils was in their Fe-diethylene triamine penta acetic acid (DTPA) content: 46.4 mg kg⁻¹ for NC and 12.7 mg kg⁻¹ for C soils.

The soils were homogenised, sieved to 4 mm, and stored in an aerated room at < 10 °C until use. The physico-chemical characteristics of the soils (Table 1) were determined by SADEF (*Société Alsacienne pour le Développement et l'Etude de la Fertilité*, Aspach-le-Bas, France). Total copper content was measured by the INRAE Soil Analysis Laboratory (LAS, INRAE, Arras, France).

2.2. *Pseudomonas putida* conservation and preparation of the inoculum

Pseudomonas putida (99.51% similar to *P. putida* ATCC 8209) originates from sediments (colonised by *Phragmites australis*) accumulated in a vineyard storm water basin (Bois et al. 2011) and was selected by Braud et al. (2015) for its tolerance to Cu and its potential ability to mobilize Cu from the soil through the production of the siderophore pyoverdine.

This bacterial isolate was green fluorescent protein (*gfp*)-tagged with pPROBE-GT-kan plasmid (Bazot et al. 2009; Miller et al. 2000,) and after addition of 0.3 mL glycerol for 1.2 mL bacterial culture, was stored at -80 °C until required.

For the experiments, cells were thawed and pre-cultivated for 48 h at 25 °C at 200 rpm in a 1:2 tryptic soy broth (TSB) medium with 50 µg mL⁻¹ of gentamicin. The cells were then transferred and cultivated for 24 h (25 °C, 200 rpm) in a larger volume of 1:2 TSB medium with 50 µg mL⁻¹ of gentamicin. Bacterial cells were centrifuged at 7,690 g, for 8 min and washed three times in KCl 9 g L⁻¹. The bacterial pellet was resuspended in a few millilitres of KCl 9 g L⁻¹ before soil bioaugmentation at the required bacterial density.

2.3. Bioaugmentation-assisted phytoextraction by sunflower

Soil and rhizobox conditioning: one week before the experiments started, the two soils (non-carbonated NC, and carbonated C) were moistened to 70% of their water-holding capacity (WHC) with ultra-pure water (ca. 12% humidity). Rhizoboxes (sides 130 mm high, 120 mm wide and 20 mm thick) were immersed in 2% HNO₃ for 24 h to remove any trace of TE and filled with 200 g dry weight (ca. 224 g wet soil), and the glass side was obscured.

Cultivation of the sunflowers in the rhizoboxes: All the rhizoboxes were planted with sunflowers (*Helianthus annuus* L. Vellox, RAGT Semences, France). Three seeds were planted in each rhizobox. One week after germination, the most developed plantlet was retained and the other two were discarded. The rhizoboxes containing the plantlets were placed on a 45° inclined stand (glass side downwards) and incubated for four weeks in a growth chamber under controlled conditions (16 h daylight at 20 °C and 8 h dark at 16 °C) 70% relative humidity and average lighting of 138 µmol of photons.m⁻² s⁻¹. The positions of the rhizoboxes in the phytotron were regularly and randomly changed.

171 *Inoculation with P. putida and rhizobox experimental setup:* Bacteria were inoculated in
172 rhizoboxes at the rate of 1.10^{12} CFU kg⁻¹ dw soil after adjustment of water-holding capacity to
173 70%. NC and C soils were bioaugmented (vs. non-bioaugmented controls) with five replicates
174 each. The rhizoboxes were bioaugmented once a week for four weeks according to Huguenot
175 et al. (2015). The first bioaugmentation took place seven days after the seeds had germinated.
176 The soil in the bioaugmented rhizoboxes was inoculated at the base of the plant stem. Control
177 rhizoboxes consisted of non-inoculated soil supplied with the same volume of KCl 9 g.L⁻¹ as
178 the bioaugmented rhizoboxes. Humidity was checked and adjusted with ultra-pure water
179 throughout the course of the experiment.

180 *Sampling and analysis:* At the end of the experiment, i.e., 31 days of sunflower culture, after
181 the removable side of the rhizobox was removed, composite samples comprising two grams
182 of wet soil were collected throughout the rhizoboxes without disturbing the soil and stored at -
183 20 °C for microbiological analysis. The soil was then removed from the rhizoboxes, and the
184 plant roots were separated from the soil. The soil adhering to the roots (averaged $117.3 \text{ g} \pm$
185 27.1) was considered as rhizospheric. Only rhizospheric soil (i.e., a 2-gram composite sample)
186 was used for analysis.

187 Plants roots were rinsed with tap water, followed by deionised water, and finally with ultra-
188 pure water and immersed in an ice-cold bath of HCl 10 mM for 10 min at 30 rpm to separate
189 the apoplastic (extracellular space) and symplastic (intracellular space) root compartments
190 (Chaignon et al. 2002). The trace elements recovered in the HCl solution were considered as
191 belonging to the apoplastic compartment whereas those accumulated in the roots were
192 considered to belong to the symplasmic compartment. After being bathed in tap water, the
193 plants were rinsed with ultrapure water. The shoots (leaves and stems) and roots (symplastic
194 compartment) were separated, weighed (fresh weight) and dried in an oven at 40 °C for five

195 days, weighed again (dry weight) then crushed and stored at room temperature until analysis.
 196 The HCl baths of roots (apoplastic solutions) were stored at 4 °C until analysis.
 197 To measure total molecular microbial biomass (MMB), microbial DNA was extracted from 1
 198 g (dry weight) of soil using a standard procedure developed by the GenoSol platform
 199 (INRAE, Dijon, France, www.dijon.inra.fr/plateforme_genosol) (Terrat et al. 2012).
 200 Concentrations of crude DNA (MMB) extracts were determined by electrophoresis in 1%
 201 agarose gel stained with GelRed® using a calf thymus DNA standard curve and used as
 202 estimates of molecular microbial biomass (Dequiedt et al. 2011). For *gfp*-tagged *P. putida*,
 203 five grams of soil were mixed with 10 mL of 1% of sodium hexametaphosphate (Braud et al.,
 204 2009; Martin 2011), incubated for 2 h at 200 rpm, and centrifuged at 500 rpm for 1 min. *gfp*-
 205 tagged cells were measured in the supernatant after dilution with a spectrofluorometer (BMG
 206 LabTech) at a wavelength excitation of 485 nm and emission of 520 nm. Calibration was
 207 performed with pure culture of *gfp*-tagged *P. putida*.
 208 Soil moisture was measured according to NF ISO 11465 (1994).
 209 Available metal concentrations were measured using CaCl₂ extractions: 5 g of soil were
 210 mixed with 10 mL of CaCl₂ (10 mM) for 2 h at 200 rpm and centrifuged at 500 rpm for 20
 211 min. The supernatant was filtered on a 0.20 µm cellulose acetate membrane and pH was
 212 immediately measured (pH-meter Mettler Toledo FE20/EL20). The absorbance of the
 213 extraction solution was measured at 380 nm as a proxy of its concentration in Pvd. This
 214 wavelength is indeed specific to Pvd since most metallophores, and more particularly those
 215 resulting from the degradation of humic substances present in the soil solution, absorb at a
 216 lower wavelength, as shown by van den Broeke et al. (2006). The concentration of Pvd in
 217 bioaugmented treatments was calculated from the difference in A³⁸⁰ values between
 218 bioaugmented and control soils and using a molar extinction coefficient (ε) equal to 13,415
 219 M⁻¹ cm⁻¹ for the two soils. The filtered supernatants were divided into two aliquots: one was

220 acidified with 1% HNO₃ 65% (v/v) for inductively coupled plasma, atomic emission
 221 spectrometry (ICP-AES) analysis and the second was preserved with 1 mM NaN₃ to measure
 222 the free ionic Cu activity (Cu²⁺).
 223 The plant samples (0.5 g dry weight) were mixed with 8 mL of HNO₃ 65 % (v/v) and 2 mL of
 224 H₂O₂ 30% (v/v) in a closed Teflon vessel. All the samples were mineralised at 175 °C for 15
 225 min in a microwave (CEM MARS 6, 1200 W). Blank and certified samples (INCT-MPH-2,
 226 Poland) were prepared for each mineralisation cycle. After digestion, the samples were
 227 cooled, recovered, and diluted to 25 mL with ultrapure water. Solutions were filtered through
 228 Whatman's No. 40 or Durieux No.111 filter paper and stored in polypropylene tubes until
 229 analysis.
 230 Total concentrations of Cu, Fe, Mn, and Zn in CaCl₂ extract, plant digests and apoplastic
 231 solutions were analysed using ICP-AES (model iCAP 6300 DUO Radial coupled with a
 232 CETAC ASX-520 auto sampler, Thermo-Fischer SCIENTIFIC, Illkirch, France), at 3,247,
 233 2,599, 2,576 and 2,062 nm, respectively.
 234 Free ionic Cu²⁺ was measured in CaCl₂ soil extracts using a cupric ion-selective electrode
 235 (ISE, 9629BNWP, Thermo Scientific Orion) according to the procedure described in Djae *et*
 236 *al.* (2017). Briefly, the ISE was calibrated daily within the 5.4 to 13.2 range of pCu (= -log₁₀
 237 a(Cu²⁺)) in Cu (CuSO₄ 0.1 mM) solutions buffered with iminodiacetic acid (IDA 1 mM) and
 238 potassium phthalate (2.5 mM). The calibration curve ($r^2 = 0.995$, $n = 12$) showed 84% slope
 239 efficiency compared with the theoretical Nernstian slope. The concentration of free ionic Cu²⁺
 240 was calculated from pCu and the Cu²⁺ activity coefficient. The latter was given by the
 241 extended Debye-Hückel equation in Ritsema (1993), assuming that the ionic strength of all
 242 the extraction solutions was fixed by the 0.01 M CaCl₂ background electrolyte.
 243 Phenotypic and physiological characterisation of the sunflower plants was performed before
 244 the plants were harvested. The length of the stems and leaves was measured, and the number

of leaves counted. Pigments can also be used to reveal the health status of plants (Young 1991). From reflectance, two indexes were calculated: (i) The normalised difference vegetation index (NDVI) is a simple and effective vegetation index that quantifies "green" vegetation relative to chlorophyll content (Datt 1998 ; Deering 1978; Ustin et al. 2009), (ii) The carotenoid reflectance index (CRI) is a pigmentation index sensitive to carotenoids present in plant foliage (Gitelson et al. 2002; Ustin et al. 2009). The equations corresponding to these indexes are as follows:

$$NDVI = \frac{R_{NIR} - R_R}{R_{NIR} + R_R}$$

$$CRI = \frac{1}{R_{510nm}} - \frac{1}{R_{550nm}}$$

where reflectance (R) was measured at the following wavelength: R_{NIR} , 800 nm (near infrared); R_R , 675 nm (red); R_{510nm} , 510 nm; R_{550nm} , 550 nm.

Hyperspectral measurements were performed on three leaves per plant, on the upper surface, using a spectroradiometer (Analytical Spectral Devices FieldSpec 4, Malvern Panalytical Ltd, Malvern, United Kingdom - Sampling step of 1 nm; Average of 10 spectra per sample; one sample corresponds to one leaf).

2.4. Data and statistical analysis

The translocation factor (TF) was determined by calculating the ratio of the concentration of metal in the shoots (mg kg^{-1}) to the concentration of metal in the roots (mg kg^{-1}).

268 XLSTAT software (Addinsoft, 2019, Paris, France) was used for two-way analysis of
269 variance (ANOVA), which examines the influence of “soil” and “bioaugmentation” in a
270 randomised multifactorial design, and p-values were determined. Principal component
271 analysis (PCA) was also performed.
272

3. Results

3.1. Dynamics of Cu

Figure 1a and Table 2 show that the amount of Cu in sunflower shoots increased ($P < 0.05$) by a factor of ca. 2 when the soil was bioaugmented, regardless of whether the soil was carbonated (C) or not (NC). Figures 1b and 1c show an increase ($P < 0.05$) in the concentration of Cu in the sunflower shoots and roots as a result of bioaugmentation. In the shoots, only the effect of bioaugmentation was significant ($P < 0.05$, Table 2) while in the roots, both the effects of bioaugmentation and of the soil were significant ($P < 0.05$, Suppl. Table T1). The concentration of copper in the shoots increased from 11.4 to 13.2 $\mu\text{g g}^{-1}$ (+16%) in the NC soil and from 13.3 to 15.6 $\mu\text{g g}^{-1}$ (17%) in the C soil (Table 2). The concentration of copper in the roots (root symplast) increased almost by a factor of 2 in the two soils, from 42.8 to 82.7 $\mu\text{g g}^{-1}$ in the NC soil and from 74.4 to 121.4 $\mu\text{g g}^{-1}$ in the C soil (Suppl. Table T1). Figure 2 shows that the fraction of root Cu present in the symplast increased ($P < 0.05$) from 1/3 to 2/3 as a result of bioaugmentation, regardless of the type of soil.

The copper translocation factor (TF, Fig. 1d) decreased significantly ($P < 0.001$) as a result of bioaugmentation.

Cu dynamics in the soil was characterized by measuring the concentration of total and free Cu (Cu^{2+}) in the CaCl_2 soil extract. Figure 1e shows that, as a result of bioaugmentation, the concentration of total Cu increased from 1.09 to 1.71 μM in the NC soil and from 1.15 to 3.00 μM in the C soil (Table 3), i.e., on average by a factor of 2.1. Conversely, Figure 1f shows that the concentration of Cu^{2+} decreased as a result of bioaugmentation, from $2.8 \cdot 10^{-2}$ to $4.0 \cdot 10^{-3}$ μM in the NC soil and from $9.8 \cdot 10^{-3}$ to $7.61 \cdot 10^{-4}$ μM in the C soil, i.e., on average by a

factor of 7 and 13, respectively (Table 3). Lastly, there was a significant effect of the soil, with a lower Cu^{2+} concentration in the C soil (Table 3).

3.2. Molecular microbial biomass and *P. putida* survival

In the controls (non-bioaugmented soils) the molecular microbial biomass (MMB) was higher in the NC soil ($17.3 \mu\text{g DNA g}^{-1}$ soil DW) than in the C soil ($8.4 \mu\text{g DNA g}^{-1}$ soil DW) (Fig. 3a). MMB increased significantly ($P < 0.001$) in the two soils as a result of bioaugmentation. MMB increased on average by a factor of 3 in the NC soil ($49.7 \mu\text{g DNA g}^{-1}$ soil DW) and by a factor of 6 in the C soil ($51.7 \mu\text{g DNA g}^{-1}$ soil DW).

Gfp-tagged *P. putida* was found at harvest in bioaugmented soils (Fig. 3b) and had increased by more than one Log unit. At harvest, the concentration of *P. putida* was significantly higher in the C soil ($7.0 \cdot 10^{13}$ CFU kg^{-1} soil DW) than in the NC soil ($4.3 \cdot 10^{13}$ CFU kg^{-1} soil DW).

3.3. Pyoverdine production and pH

The absorbance of the extraction solution at 380 nm, used as a proxy of the concentration of Pvd, increased on average by a factor of 2.3 as a result of bioaugmentation (Table 3). Pvd production in bioaugmented soils led to a concentration of about $1.5 \mu\text{M}$ in the CaCl_2 extract. The pH of the CaCl_2 extract only slightly decreased with bioaugmentation (from 6.81 to 6.57; see Table 3), although the effect of bioaugmentation was statistically significant ($P < 0.05$). However, the main difference in pH was observed between the two soils.

3.4. Plant growth and fitness

A significant effect of bioaugmentation ($P < 0.05$) was observed on almost all the biometric parameters of sunflower (Table 4). Plants grown on bioaugmented soils produced on average more biomass (plant dry mass increased 1.3 to 1.6-fold), more leaves (3 to 4 more leaves) and

longer leaves (on average 1.3- to 1.4-longer than plants grown on the control C and NC soils). Conversely, the stem was on average 15% shorter ($P < 0.05$) in plants grown on both bioaugmented C and NC soils as well as the ratio of root to shoot biomass (RS), but only those grown on the NC soil. In the controls, the dry mass of plants was significantly higher ($P < 0.05$) when grown on the NC soil than on the C soil. Table 4 also shows that the two indexes (NDVI and CRI) were higher ($P < 0.05$) in plants grown on bioaugmented soils, suggesting better plant fitness under this treatment. It is worth noting that the increase in CRI caused by bioaugmentation was more pronounced than that in NDVI, and that both indexes increased to the same extent in the two soils (NC and C).

3.5. Dynamics of Fe, Mn, Zn, and P

Both the concentration and the amount of Fe, Mn, Zn, and P in the sunflower shoots increased significantly ($P < 0.05$) as a result of bioaugmentation (Table 2). The amount of Fe, Mn, Zn, and P in sunflower shoots increased by respectively a factor of 1.5, 3.3, 1.1 and 1.7 in the NC soil, and 2.4, 5.3, 1.8 and 2.7 in the C soil. In the controls, the amount of Mn, Zn, and P in sunflower shoots was significantly higher ($P < 0.05$) in the NC soil than in the C soil. The concentration of Fe, Mn, Zn, and P in sunflower shoots increased by respectively a factor of 1.9, 1.5, 1.1 and 1.5 in the NC soil, and 2.8, 2.2, 1.6 and 2.2 in the C soil. For Zn this increase did not exceed 15% and was only significant ($P < 0.05$). The difference in the root elemental composition between the two soils was more pronounced. Notably, the concentration of Mn in the roots was on average 4-fold higher ($P < 0.05$) in the C soil than in the NC soil, regardless of the treatment. As a consequence, the amount of Mn in the roots was on average 3- to 5-fold higher in the NC soil than in the C soil, depending on the treatment. Table 3 shows a significant effect ($P < 0.05$) of bioaugmentation on the concentration of Fe in the CaCl_2 extract (1.1 to 1.3-fold increase depending on the soil) and Mn (2.3- to 3.1-fold

increase depending on the soil), but not Zn. The effect of bioaugmentation was more pronounced for P, whose concentration in the CaCl₂ extract was on average 8 to 9-fold higher in the bioaugmented treatments than in the controls. A significant effect ($P < 0.05$) of the type of soil was also observed: the concentration of Fe, Mn, and Zn was higher in the NC soil than in the C soil.

3.6. Multivariate analysis of all the parameters monitored in the CaCl₂ extract

Principal component analysis (PCA) of the parameters monitored in the CaCl₂ extract (Suppl. Fig. F2) reveals positive correlations between the concentrations of total Cu (Cu_{TOT}), Fe, Mn, P, and Pvd, as well as with the molecular microbial biomass (MMB) and *P. putida* as supplementary variables. pH was mostly negatively correlated with Zn (and to a lesser extent with Fe and Mn) whereas no correlation was observed between pH and the concentration of Cu_{TOT} and Cu_{FREE} (both negatively correlated). PCA clearly separates into two clusters according to the soil (C and NC). It also distinguishes the treatment, i.e., whether the soils were bioaugmented or not (in particular NC soil). This emphasizes the fact that the overall effect of bioaugmentation is to increase the total concentrations of elements in the CaCl₂ extract (and to decrease the concentration of Cu²⁺).

4. Discussion

4.1. Effect of bioaugmentation with *Pseudomonas putida* on the phytoextraction of Cu (and other elements)

The amounts of Cu extracted by the aerial parts of sunflower (Fig. 1a, Table 2) increased by up to 87% in soils bioaugmented with *P. putida*, i.e., in line with the few studies conducted on

this TE (Andreazza et al. 2010; Chen et al. 2005; Huguenot et al. 2015; Ju et al. 2019; Yang et al. 2013), with copper concentrations in soils in the same range as in our study (50 up to 1,230 mg Cu kg⁻¹ soil). It is worth noting that this increase was also observed for Fe, Mn and P (Table 2).

The improved performance of phytoextraction with bioaugmentation is largely due to better sunflower growth (30% to 60% increase, Table 4) with our *P. putida* (well-known PGPR). Numerous studies have demonstrated the effect of PGPR on plant growth (for reviews, see Goswami et al. 2016, Vacheron et al. 2013, Vejan et al. 2016). The production of complexing agents including pyoverdine is one of the well-known properties of PGPR (Saharan and Nehra 2011) with, e.g., the plant's ability to obtain more iron, as shown in our study, with an increase by a factor of 1.5 up to 1.9 (Table 2).

The yellowing of leaves for non-bioaugmented plants (Suppl. Fig. F3) did not result of the phytotoxic effect of copper since copper-CaCl₂ (Table 3) concentrations in the non-carbonated vineyard soil (NC) and the carbonated one (C) were 1.09 and 1.15 µM respectively, i.e., well below the concentration (10 µM) defined by Garcia et al. (1999) as toxic for sunflower and as causing oxidative stress. Besides shoot Cu was within the normal range, i.e., 3-30 µg Cu g⁻¹ DW (Marschner 2012) (Fig. 1b and Table 2). Although the concentrations of iron in the shoots of sunflower grown on both control soils were lower than those measured in the bioaugmented soils (Table 2), the diffuse yellowing observed did not look like that of iron deficiency, which is characterised by discolouration of the veins. Besides, the iron concentrations measured in the shoots (Table 2) were within the normal range (Marschner 2012). Therefore, we suspected yellowing to be related to nitrogen deficiency. The fact that plants grown on control soils allocated a relatively higher fraction of their dry matter to the roots (higher RS) than plants grown on bioaugmented soils (Table 4) may also indicate such a nitrogen deficiency (Wang et al. 2014). According to Agüera et al.

(2010), such nutritional stress can cause leaf senescence in sunflower. The uniform chlorosis of the vegetative part of the older leaves was also reported by Alves et al. (2019) in sunflower. Lastly Agüera et al. (2010) demonstrated that total chlorophyll content and the photosynthetic rate of sunflower decreased with a low level of N (2 μ M). Nitrogen was not measured in our study, but the overall photosynthetic activity as shown by the two indexes was lower in the control soils (Table 4). While NDVI values can vary from 0 to +1.0, the high values in Table 4 (0.86 to 0.9) are typical of mature plants. They are close to those reported by Turhan et al. (2008) for sunflowers grown in the laboratory in sand pots, or by Tunca et al. (2018) for sunflowers grown in the field. It should be noted that the positive relationship between NDVI and leaf size was already demonstrated, especially for sunflower, by Wanjura and Hatfield (1986). Carotenoids also play an important role in photosynthesis as accessory pigments, but their content can also increase with light stress or with plant senescence (Adams et al. 1996, Ustin et al. 2009, Ustin and Jacquemoud, 2020). This was not the case in our experiment, where all plants were young and grown under optimal growth conditions, especially lighting. Hence the values obtained (5.5 to 8.4) on a scale of 1 to 12 are commonly accepted for green plants. The significantly higher CRI values for plants cultivated on bioaugmented soils (7.1 and 8.4) compared to control plants (5.5 and 5.8) indicate, like for NDVI, better plant development.

In addition to the typical effect of PGPR on the plant growth, an increase in Cu concentration in aerial parts was also observed – both effects contributing to the higher amount of Cu extracted by plants –as shown in several studies (Abou-Shanab et al. 2008; Almeida et al. 2017; Andreadza et al. 2010; Brunetti et al. 2012; Ju et al. 2019; Ma et al. 2009; Mendoza-Hernández et al. 2019; Płociniczak et al. 2013). This indicates an increase in the soil-plant transfer rate of copper. The increase in Cu mobility in the soil with bioaugmentation (by a factor of 1.6 to 2.6) explains the improved phytoextraction performance (the same

conclusions apply to Fe, Mn and P). Since complexed forms of Cu predominate in the soil solution to the detriment of Cu^{2+} (Fig. 1e, f), this suggests that the plant took up Cu in complexed form in addition to Cu^{2+} , or that a decomplexation took place during uptake by the roots. It is also possible that partially degraded Pvd complexed with Cu was more easily internalised by sunflower. Cornu et al. (2014) already showed that adding Pvd to a calcareous soil significantly increased the uptake of Cu by barley and tomato, thereby suggesting the contribution of Cu-Pvd to Cu uptake by plants. Although the free-ion-activity model (FIAM) is the most commonly used model (Campbell, 1995), models derived from the FIAM have also been reported in which complexes also affected TE uptake by plant roots (for a review, see Nowack et al., 2006). These models included the dissociation of TE complexes within the diffusion layer (Degryse et al., 2006) and/or from the passive uptake by plant roots of non-dissociated TE complexes, such as TE-EDTA (Schneider et al., 2006). Shirley et al. (2011) and Vansuyt et al. (2007) even showed that plants were able to incorporate iron from Fe-Pvd and, because Pvd was detected *in planta*, they proposed that Fe-Pvd was at least partially incorporated in a non-dissociated form by plant roots.

Few studies have compared the Cu translocation factors (TF) in bioaugmented and control soils. Comparing studies is tricky because results depend on how the roots are prepared before the determination of Cu. Considering only symplasmic Cu, i.e., the intracellular compartment (Chaignon et al. 2002), probably explains why TF values (Fig. 1b) decreased with bioaugmentation. In contrast, when Cu was summed in the apoplasmic and symplasmic compartments, TF increased with bioaugmentation as observed by Abou-Shanab et al. (2008) and Mendoza-Hernandez et al. (2019). In any case, the TF values we measured in the present study are in the range of those reported in the literature. The transfer of Cu from the apoplasmic to the symplasmic compartment can be explained by the degradation of cell walls

by bacterial siderophores, as already reported by Hazotte et al. (2018), with signs of root necrosis (blackish appearance) following the addition of 50 μ M Pvd.

4.2. Establishment of *P. putida* in the soil and role played by the siderophore pyoverdine in the mobilization and phytoavailability of copper (and other trace elements)

The mechanisms governing the mobilization of Cu from the soil and its uptake by the plant, following bioaugmentation by a bacterium selected for its siderophore production potential (Braud et al. 2015), have never been described. Previous studies selected microorganisms potentially able to improve phytoextraction (Bouquet et al. 2020 for Pb; Jalali et al. 2020 for Cd, Sr, and some rare earths, Durand et al. 2016 for Ni), however without an in-depth investigation of the underlying mechanisms. Almost all studies fail to describe the succession of processes: survival of the inoculated bacterial population, production of the metabolite of interest (in our case, pyoverdine) and its effects on the mobilization and uptake of TE by the root. Regarding bacterial survival, different inocula densities and re-inoculation frequencies, as well as inoculum conditioning and nutrient supply, have been tested (for a review, see Lebeau 2011), but very few studies have monitored inoculum survival (Braud et al. 2009; Trevors et al. 1993). Huguenot et al. (2015) showed that implantation of the strain in the soil required four weekly reinoculations before the inoculated strain became established. This is why we reinoculated the soil at this frequency (Fig 3b) to be sure that *P. putida* was properly established in the soil. The proportion of *P. putida* in the molecular microbial biomass (MMB) was higher in the carbonated (C) soil (Fig 3), which could be explained by the fact that pH was, as expected, 1 unit higher than in the non-carbonated (NC) soil (7.8 vs. 6.7). Indeed, the optimum pH for the growth of *P. putida* is 7-7.5 (Elsayed and El-Nady 2013). A difference in soil structure due to the difference in texture (the C soil is sandier and richer in CaO than the

NC soil, but less clayey), and also in nutrient availability could explain the difference in soil colonisation by *P. putida* (Table 1). Lastly, the difference in sunflower growth as a function of the soil (Table 4) most certainly modulates microbial abundance and composition, due to a difference in the quantity and composition of rhizodeposits (Bardgett et al. 1999; Newman and Watson 1977).

The increase in absorbance (A^{380}) in the CaCl_2 extract between the control (CONTROL) and the bioaugmented modality (BIOAUG) was mostly in Pvd (Table 3) as already shown in a previous study where purified Pvd was directly added to a collection of vineyard soils (Cornu et al. 2019). The increase in Pvd production when the soil is bioaugmented is attributable to the presence of *gfp*-tagged *P. putida* (Fig. 3b, Suppl. data F2), suggesting a direct effect of bioaugmentation. But other microorganisms, in particular other *Pseudomonas* that are well represented in the rhizosphere of most plants (Lugtenberg et al. 2001) may also be involved in additional Pvd production since the Pvd concentration also increased with MMB (Fig. 3a). Thus, an indirect effect of bioaugmentation cannot be excluded. With the assumption that the increase in A^{380} between CONTROL and BIOAUG only results from Pvd production, this corresponds to a Pvd concentration of more than 2 nmol/g soil, which is 20 to 2 000 times higher than the siderophore concentrations measured by Boiteau et al. (2018) and Rai et al. (2020). This discrepancy is not surprising given the densities of *P. Putida* and the repetition of the inoculations in our study. Yet the Pvd concentrations we measured in the CaCl_2 extract represent only a small fraction of the Pvd produced, which was most likely sorbed onto the soil carrier phases, as shown by Cornu et al. (2019), i.e., 73-96% of the Pvd added to a collection of vineyard soils. We would therefore expect to find 9 to 67 μM of Pvd, which is consistent with the minimum concentration of Pvd (between 25 and 50 μM) added to a calcareous soil to significantly mobilize Cu (Cornu et al. 2014).

In bioaugmented soils (BIOAUG), Cu concentration in the soil solution increased by a factor of 1.57 (NC) to 2.63 (C) (Table 2). Cornu et al. (2019) obtained an average increase of a factor of 4 (from 1.4 to 8 depending on the vineyard soil studied), but with the addition of 200 $\mu\text{mol Pvd kg}^{-1}$ of soil, suggesting that increased Pvd production by bioaugmentation could enhance Cu mobilization. This was also demonstrated in a previous study (Cornu et al., 2014) in which the mobility of Cu in a calcareous soil increased exponentially with an increase in the dose of Pvd supplied.

The higher concentration of Cu in the CaCl_2 extract when the soils were bioaugmented (Fig 1e) is due to its mobilization from the soil bearing phases. This correlates with the increase in Pvd concentration (Suppl. data Fig 2) as already demonstrated by Cornu et al. (2019) in a collection of vineyard soils. It is worth noting that there was no effect of the soil (C vs. NC) which is consistent with Pvd whose production did not depend on the soil. Like in the study by Cornu et al. (2019), in which Pvd was directly added to the soil, the slope close to one (Suppl. data Fig 1a) and the close to zero intercept of the regression line suggest that all the Pvd was complexed with Fe and Cu (Al non-detectable) in the CaCl_2 extract. This result shows that Fe and Cu were mobilized by complexolysis by Pvd (siderophore known to form 1:1 M complexes) in bioaugmented soils. Strengthening this hypothesis concerning the major role of complexolysis, we observed that the pH of the CaCl_2 extract was not affected when the soils were bioaugmented (Suppl. data Fig 2: see PCA), like Cornu et al. (2019) who added Pvd directly to their collection of vineyard soils. Although lower than that of iron ($K^{\text{L/Fe}} = 10^{30.8}$), the stability constant of Pvd-Cu ($K^{\text{L/Cu}} = 10^{20.1}$) for *Pseudomonas aeruginosa* which is much higher than that of Cd ($K^{\text{L/Cd}} = 10^{8.2}$) (Cornu et al. 2014) explains why Cu in addition to Fe is mobilized by complexolysis from the soil-bearing phases.

However, like in the study by Cornu et al. (2019), bioaugmentation reduced the concentration of Cu^{2+} in the soil solution. The reduction in the Cu^{2+} concentration from a factor of 7 (NC

soil) to 12.8 (C soil), is consistent with the results of Cornu et al. (2019) (from a factor of 2 to 37) in which Pvd was directly added to the soil.

Mn showed very similar behaviour to that of Fe (Suppl. data Fig. 2). It was even more strongly correlated with Pvd than Cu and Fe. Duckworth and Sposito (2007) and Harrington et al. (2012a) highlighted the complexation capacities of Mn (III) by Pvd with the Log of the stability constant for Pvd-Mn(III) (35.3-35.4) higher than that of Pvd-Fe (30.0-32.4) (Harrington et al. 2012b), which probably also explains the higher level of Mn extraction following bioaugmentation ($\times 3.1$ for NC soil vs. $\times 2.3$ in the C soil (Table 3)) compared to that of Fe ($\times 1.3$ and $\times 1.13$ respectively). One could hypothesize that Mn is also mobilized by ligand-controlled dissolution, together with Fe and Al, although Suppl Fig.1b shows a positive and close relationship with a slope close to 3 which is not consistent with what should be obtained with Pvd, which forms 1:1 complexes. This suggests that the Mn mobilized by Pvd is mobilized by a reductive dissolution process allowing recycling of Pvd, as shown by Akafia et al. (2014) in a batch dissolution experiment. Indeed, in reductive dissolution, the recycling of the mobilizing ligand (here Pvd) enables mobilization of the metals with a ratio of mobilized metals to Pvd in solution higher than 1.

The concentration of Pvd in the bioaugmented soil did not vary significantly, whether the soil was carbonated or not ($\times 2.3$ and $\times 2.4$ respectively (Table 3)), a trend that is similar to the performance of bioaugmentation-assisted phytoextraction (increase by a factor of 1.9 and 1.7 respectively, in C and NC soils). This result goes against our initial hypothesis. Indeed, with the C soil, where iron is less available than with the NC soil (12.7 mg Fe-EDTA kg⁻¹ or 228 μ moles kg⁻¹ vs. 46.4 mg kg⁻¹ or 831 μ moles kg⁻¹; Table 1), one could have expected a higher Pvd concentration, as demonstrated by Persson et al. (1990). Most authors indeed report iron concentrations that stimulate vs. repress Pvd production, for example, Sayyed et al (2005) who demonstrated that maximum production of Pvd by fluorescent Pseudomonads was

obtained at 1 μM of Fe and repressed from 30 μM , and Parker et al. (2007) who showed repression of Pvd production by *Pseudomonas putida* GB-1 from 2 μM FeSO_4 . But if we consider the Fe concentrations in the CaCl_2 extract (bioavailable fraction) of the two soils, they were not very different (2.0 μM for soil C vs. 2.2 μM for NC; Table 3), which may explain the similar Pvd concentrations in the C and NC soils and hence the absence of a soil effect when bioaugmented. Thus, CaCl_2 extraction seems more suitable than EDTA extraction to assess the effect of iron on Pvd production. Another explanation for the similar production of Pvd suggested by Marschner and Crowley (1997) is that the concentration of iron (5 vs. 50 mM FeCl_3) has no effect on Pvd production. These authors postulated that the strain of *P. fluorescens* Pf-5(pvd-inaZ) they used utilized siderophores produced by other microorganisms or by plants.

Bioaugmentation resulted in additional Zn mobilization only in the C soil (Table 3). Zinc is negatively correlated with pH (no correlation with Pvd) indicating that Zn is mobilized mainly by ion exchange, in agreement with the results of previous works (Al-Wabel et al., 2002; Cornu et al., 2017, Cornu et al. 2019). The slight variation in soil pH certainly explains the absence of a significant effect of bioaugmentation on additional Zn mobilization (Table 3). In the end, the very high P mobilization following bioaugmentation ($\times 7.7$ for NC soil vs. $\times 8.6$ for C soil) (Table 3) with a high correlation between the concentration of P- CaCl_2 and Pvd- CaCl_2 (Suppl. data Fig. 2) appears to reveal one of the possible mechanisms of P solubilisation by siderophores (Sharma et al. 2013), although other more widespread mechanisms exist, in particular the action of phosphatases. Our *P. putida* strain was selected for its ability to produce phosphatases (Braud et al. 2015).

5. Conclusion

Bioaugmentation with *P. putida* increased the total concentration of Cu in the soil solution up to 2.6-fold and we showed that this increase was correlated with both the density of *gfp*-tagged *P. putida* and the surplus of pyoverdine produced by *P. putida* (and possibly by other native *Pseudomonas* populations in the soil). The concentration of Cu^{2+} was markedly reduced (by up to a factor of 13) due to its complexation by Pvd (and most probably other ligands) in the soil solution. However, the concentration and amount of Cu in the sunflower plants increased, which raises the question of the mechanisms of Cu uptake by the plant (internalisation of the complex and/or decomplexation in the rhizosphere and uptake in the form of Cu^{2+}).

To achieve the required level of phytoextraction (to compensate for the maximum annual average of 4 kg Cu ha⁻¹), promoting the decomplexation of Pvd-Cu and more broadly of the Cu-ligands present in the soil solution should be explored by adjusting the pH but also by promoting the degradation of complexing agents.

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Figure captions

Figure 1. Copper dynamics at the soil-plant interface in two vineyard soils (NC for non-carbonated and C for carbonated) cultivated for 31 days with sunflower (cv. *Velox*) and bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*: (a) amount of Cu in shoots, (b) concentration of Cu in shoots, (c) concentration of Cu in roots, (d) root-to-shoot translocation of Cu, (e) total concentration of Cu in 0.01 M CaCl₂ extract, and (f) free ionic concentration of Cu in 0.01 M CaCl₂ extract. For each soil, (*), *, ** and *** indicate that the average value measured in the bioaugmented treatment differed significantly from the one measured in the control, at the probability level of $P < 0.1$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. The error bars stand for mean standard deviations and ns stands for non-significant.

Figure 2. Fraction of root Cu stored in the symplast in sunflower (cv. *Velox*) grown for 31 days on two vineyard soils (NC for non-carbonated and C for carbonated) bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*. For each soil, *** indicate that the average value measured in the bioaugmented treatment differed significantly from the one measured in the control, at the probability level of $P < 0.001$. The error bars stand for mean standard deviations and ns stands for non-significant.

Figure 3. Total microbial biomass (a) and gfp-tagged *Pseudomonas putida* (b) determined in soil adhering to the roots (i.e. rhizospheric soil) after 31 days of sunflower (cv. *Velox*) cultivation in two vineyard soils (NC for non-carbonated and C for carbonated) bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*. In (a), for each soil, ** and *** indicate that the average value measured in the bioaugmented treatment differed significantly from the one measured in the control, at the probability level of $P < 0.01$ and $P < 0.001$, respectively. In (b), (*) indicate that the average value measured in the soil C differed significantly from the one measured in the soil NC, at the probability level of $P < 0.1$. The error bars stand for mean standard deviations and ns stands for non-significant.

Figure 1

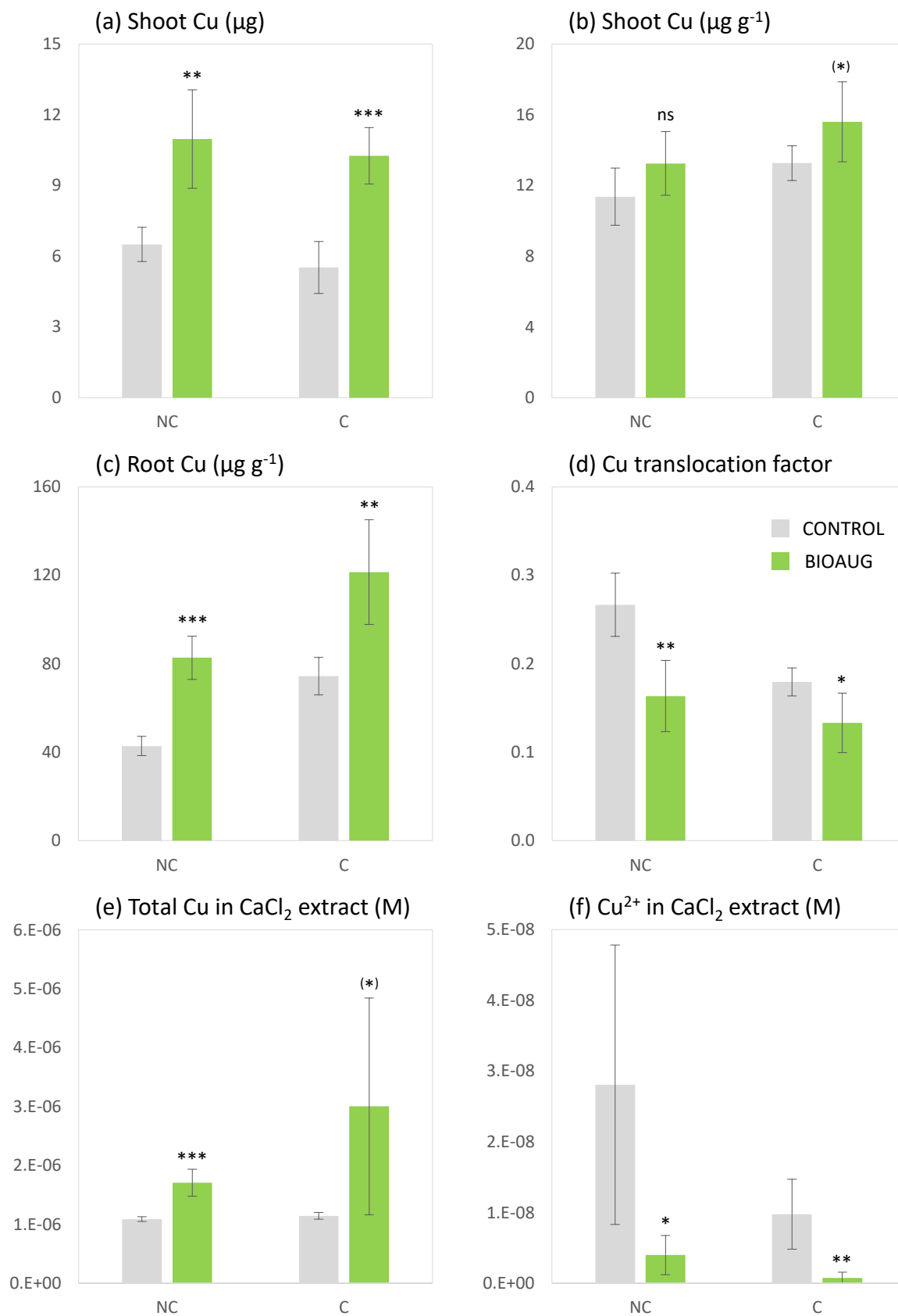


Figure 2

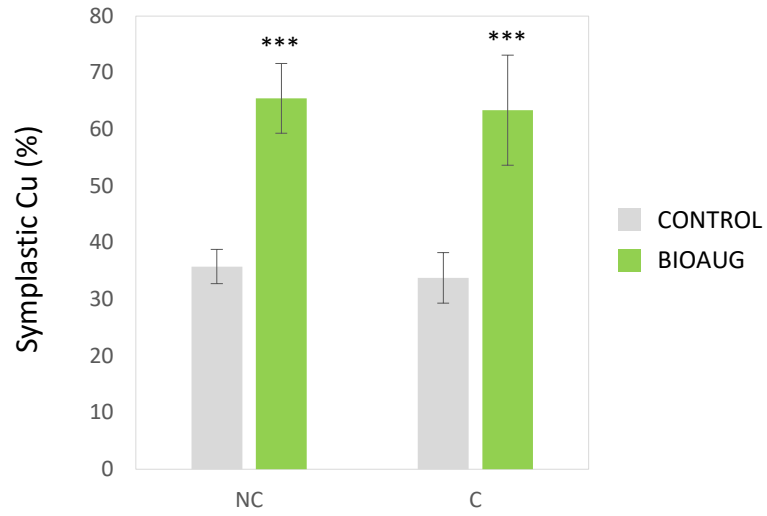
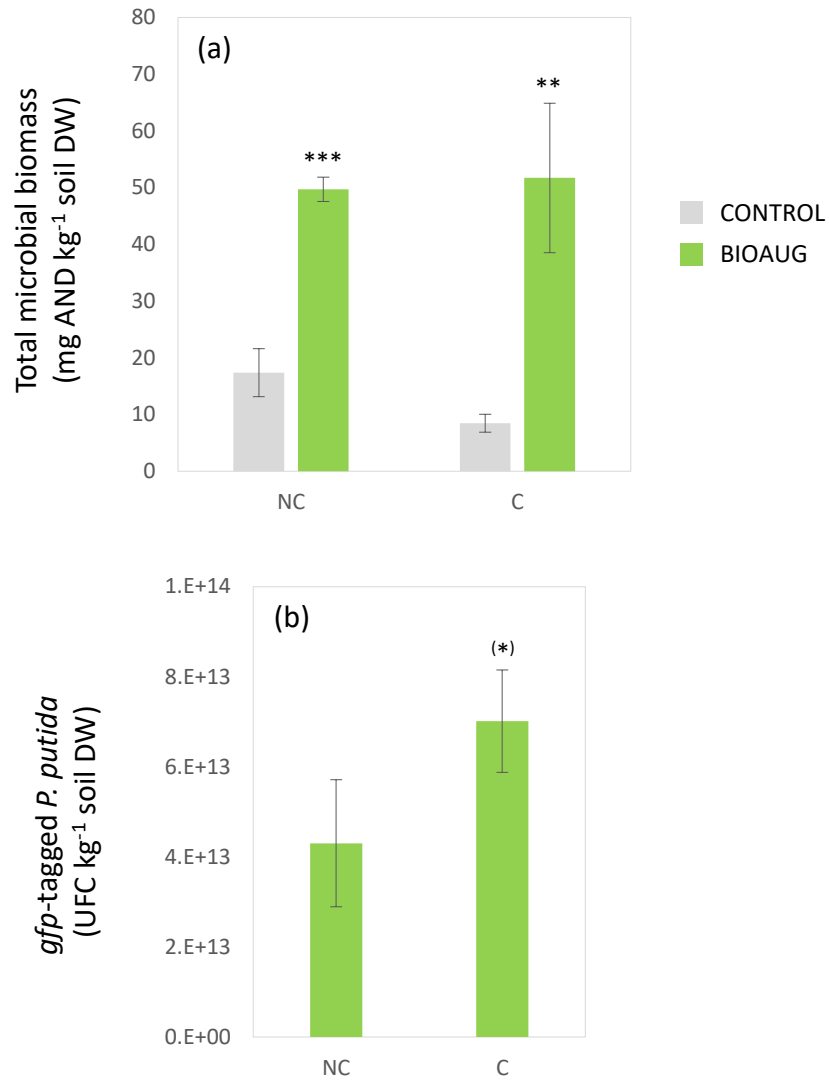


Figure 3



1 **Table 1.** Physico-chemical characteristics of the non-carbonated (NC) and the carbonated (C)
2 soils used in the study. Results are expressed on a soil dry weight basis.

Parameters	Units	NC	C
Clay	g kg ⁻¹	84	44
Fine silt	g kg ⁻¹	45	43
Coarse silt	g kg ⁻¹	46	41
Fine sand	g kg ⁻¹	94	134
Coarse sand	g kg ⁻¹	731	738
pH _{water}	-	6.7	7.8
Total carbonates	g kg ⁻¹	< 0.1	9
Organic matter	g kg ⁻¹	44.6	43.5
C:N ratio	-	11.6	12.5
Cationic exchange capacity (Metson method)	me kg ⁻¹	73	33
P ₂ O ₅ (Dyer method)	g kg ⁻¹	0.4	0.4
DTPA-Cu	mg kg ⁻¹	40.2	50.9
DTPA-Fe	mg kg ⁻¹	46.4	12.7
DTPA-Mn	mg kg ⁻¹	5.8	3.5
DTPA-Zn	mg kg ⁻¹	6.4	10.7
Total Cu (HF)	mg kg ⁻¹	127	174

3

4

Table 2. Concentration and corresponding amounts of Cu, Fe, Mn, Zn and P in shoots of sunflower (cv. *Velox*) grown for 31 days on two vineyard soils (NC for non-carbonated and C for carbonated) bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*.

Soil	Treatment	Concentration in plant shoots					Amount in plant shoots				
		Cu	Fe	Mn	Zn	P	Cu	Fe	Mn	Zn	P
		$\mu\text{g g}^{-1}$ DW				%	μg				mg
NC	CONTROL	11.4	45.7	<u>44.1</u>	62.2	0.28	6.5	26.0	<u>25.2</u>	<u>35.5</u>	<u>1.59</u>
	BIOAUG	13.2 ^{ns}	88.6 ^{**}	68.0 ^{**}	69.0 ^{ns}	0.43 ^{**}	11.0 ^{**}	73.1 ^{***}	56.2 ^{***}	<u>57.0</u> ^{**}	3.56 ^{***}
C	CONTROL	13.3	<u>68.6</u>	26.8	60.9	0.27	5.5	28.3	11.2	25.3	1.11
	BIOAUG	15.6 ^(*)	104 ^{***}	<u>89.6</u> ^{***}	68.7 [*]	0.46 ^{**}	10.3 ^{***}	68.5 ^{***}	59.0 ^{***}	45.3 ^{***}	3.03 ^{***}
ANOVA	SOIL	*	**	ns	ns	ns	ns	ns	*	***	**
	BIOAUG	*	***	***	*	***	***	***	***	***	***
	SOIL*BIOAUG	ns	ns	***	ns	ns	ns	ns	**	ns	ns

Values are means of five independent replicates

Two-way ANOVA was performed to assess the effects of the soil and the bioaugmentation with *P. putida* on the concentration and the corresponding amount of metals and P recovered in plant shoots: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively

For a given soil, (*), *, ** and *** indicate that the mean value in the inoculated treatment differed significantly from that in the control, at a probability level of 0.1, 0.05, 0.01 and 0.001, respectively

For a given treatment, mean values are underlined when they are significantly higher ($P < 0.05$) than that measured in the other soil.

Table 3. pH, A³⁸⁰ and concentrations of pyoverdine (Pvd), Fe, Mn, Zn, P, total Cu (Cu_{tot}) and free ionic Cu (Cu²⁺) in 0.01 M CaCl₂ extract from two vineyard soils (NC for non-carbonated and C for carbonated) bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*. Soil extraction was made from soil adhering to the roots (i.e. rhizospheric soil) after 24 days of sunflower (cv. *Velox*) cultivation.

Soil	Treatment	pH	A ³⁸⁰	Pvd μM	Fe	Mn	Zn	P	Cu _{tot}	Cu ²⁺
NC	CONTROL	5.68	0.014	-	<u>2.19</u>	<u>1.57</u>	<u>2.12</u>	<u>12</u>	1.09	2.81 E-2
	BIOAUG	5.70 ^{ns}	0.032 ^{***}	1.56	<u>2.91</u> ^{**}	<u>4.82</u> ^{***}	<u>2.16</u> ^{ns}	94 ^{***}	1.71 ^{***}	<u>4.00 E-3</u> [*]
C	CONTROL	<u>6.81</u>	0.015	-	1.98	0.94	1.56	10	1.15	9.77 E-3
	BIOAUG	<u>6.57</u> ^{**}	0.036 ^{***}	1.35	2.23 [*]	2.13 [*]	1.72 [*]	90 ^{**}	3.00 ^(*)	7.61 E-4 ^{**}
ANOVA	SOIL	***	ns	-	***	***	***	ns	ns	*
	BIOAUG	*	***	-	***	***	ns	***	**	**
	SOIL*BIOAUG	*	ns	-	*	*	ns	ns	ns	ns

Values are means of five independent replicates

Two-way ANOVA was performed to assess the effects of the soil and the bioaugmentation with *P. putida* on the pH and the concentrations Pvd, metals and P measured in CaCl₂ extracts: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively

For a given soil, (*), *, ** and *** indicate that the mean value in the inoculated treatment significantly differed from that in the control, at the probability level of 0.1, 0.05, 0.01 and 0.001, respectively

For a given treatment, mean values are underlined when they are significantly higher (P < 0.05) than that measured in the other soil.

Table 4. Biometric parameters (biomass, ratio of root to shoot biomass (RS), stem length, average leaf length and number of leaves) and fitness (NDVI and CRI indexes) of sunflower (cv. *Velox*) grown for 31 days on two vineyard soils (NC for non-carbonated and C for carbonated) bioaugmented (BIOAUG) or not (CONTROL) with *Pseudomonas putida*.

Soil	Treatment	Plant biometrics					Plant fitness	
		Plant biomass mg DW	RS	Stem length cm	Average leaf length cm	Number of leaves	NDVI	CRI
NC	CONTROL	<u>808</u>	0.41	14.5	4.0	7.4	0.86	5.5
	BIOAUG	1066 ***	0.29 **	12.3 *	5.3 ***	10.8 ***	0.88 *	7.1 *
C	CONTROL	616	0.49	14.0	3.6	6.4	0.86	5.8
	BIOAUG	978 ***	<u>0.48</u> ns	11.9 **	5.2 ***	10.6 ***	<u>0.90</u> **	8.4 *
ANOVA	SOIL	***	**	ns	ns	ns	ns	***
	BIOAUG	***	ns	***	***	***	***	***
	SOIL*BIOAUG	ns	ns	ns	ns	ns	ns	*

Values are means of five independent replicates

Two-way ANOVA was performed to assess the effects of the soil and the bioaugmentation with *P. putida* on the biometric parameters and the fitness of sunflower: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively

For a given soil, (*), *, ** and *** indicate that the mean value in the inoculated treatment differed significantly from that in the control, at a probability level of 0.1, 0.05, 0.01 and 0.001, respectively

For a given treatment, mean values are underlined when they are significantly higher ($P < 0.05$) than that measured in the other soil.



Cu phytoextraction by 87%

