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REGULAR ARTICLE

Anthyllis vulneraria/*Mesorhizobium metallidurans*, an efficient symbiotic nitrogen fixing association able to grow in mine tailings highly contaminated by Zn, Pb and Cd

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Abstract The excessive concentrations of toxic heavy metals in mine tailings and their very low N content make soil reclamation strategies by phytostabilization difficult. Our objective was to test if the symbiotic association between the legume *Anthyllis vulneraria* subsp. *carpatica* and the bacteria *Mesorhizobium metallidurans* originating from highly polluted mine tailings is able to increase N concentration in soils with contrasting Zn, Pb and Cd contents. Plants of *A. vulneraria* subsp. *carpatica* from a mine site and of a non-metallicolous subsp. *praeopera* from non-polluted soil were inoculated with a metallicolous or a non-metallicolous compatible *Mesorhizobium* spp. and grown on low and high heavy metal-contaminated soils. In contaminated soil, many nodules were observed when the metallicolous *A. vulneraria* was inoculated with its rhizobium species *M. metallidurans*, whereas the non-metallicolous *A. vulneraria* died after a few weeks regardless of the rhizobium inoculant. Eighty percent of the total nitrogen was derived from biological nitrogen fixation through the association between metallicolous *A. vulneraria* and the rhizobium grown on metal-enriched soil. The ability of the metallicolous *A. vulneraria* to develop a high nitrogen fixing potential opens new possibilities for promoting a low-maintenance plant

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cover and for stabilizing the vegetation in high heavy metal-contaminated soils.

Keywords Nitrogen fixation · Legumes · *Rhizobium* · Heavy metals · Metallophytes · Phytostabilization · Metal tolerance

Introduction

Soils in mining areas and around smelters are enriched with toxic heavy metals such as Zn, Pb and Cd and they have a very low organic matter and nitrogen (N) content. Plant species diversity is drastically limited since few plant species are able to grow on such soils (Ernst 1990). These areas are sources of serious environmental and health hazards and strategies such as phytostabilization to immobilize toxic compounds have been increasingly developed in the last decade (Salt et al. 1998). For the ecological and economic rehabilitation of mine tailings, we need to find plant species tolerant to heavy metals that would serve as a direct and indirect source of nutrients and facilitate the installation of other species to increase biological diversity (Ernst 1996). Phytostabilization of contaminated soils after industry or mining activities may be considerably improved by introducing legumes as pioneer plants (Rastetter et al. 2001; Frérot et al. 2006; Khan et al. 2009). Mutualistic interactions between legumes and their symbiotic rhizobium bacteria play a key role in ecosystem restoration mainly by increasing plant productivity through enhanced N capture and N_2 fixation (Parker 1995; Vitousek and Field 1999; Van der Heijden et al. 2006) and by promoting the maintenance of plant cover (Harris et al. 1996; Whiting et al. 2004). However, high concentrations of Zn and Cd can either restrict the nodulation of legume species (El-Kenawy et al. 1997; Chaudhary et al. 2004) or inactivate biological symbiotic nitrogen fixation (BNF) by forming ineffective root nodules with the rhizobium (McGrath et al. 1988). Therefore, examples showing symbiotic associations conserving their ability to fix N on highly contaminated soils are scarce (Giller et al. 2009). A white clover (*Trifolium repens*) population was identified on soil containing up to 20,000 mg Zn kg^{-1} soil and 30,000 mg Pb kg^{-1} soil (Rother et al. 1982), and more recently symbiotically effective *Sinorhizobium meliloti* strains tolerant to As, Cu and Pb were found in soils contaminated after the toxic spill at the Aznalcollar pyrite mine (Carrasco et al. 2005). Free-living bacteria were isolated from mine tailings and *Cupriavidus* (formerly *Wautersia/Ralstonia) metallidurans* was described as a model metal-tolerant bacterium (Mergeay et al. 2003; Vaneechoutte et al. 2004).

Most of the measurements of symbiotic N_2 fixation in heavy metal-contaminated soils have concerned cultivated leguminous plants growing in soils amended with sewage sludge where heavy metal concentrations were far lower than those encountered in mine tailings (Obbard and Jones 2001; Broos et al. 2004; Chaudri et al. 2008).

The identification and selection of metal-resistant symbiotic N_2-fixing plant-bacterium associations is a crucial aspect for overcoming the prime constraints of N input and organic matter enhancement of mine tailings. In this context, Frérot et al. (2006) identified a plant species *Anthyllis vulneraria* subsp. *carpatica* growing in a highly contaminated soil in the Les Avinières mine (South of France). The symbiotic partner of *Anthyllis vulneraria* was isolated from a nodule of the host plant and was identified as a single, novel species *Mesorhizobium metallidurans* (Vidal et al. 2009). When cultivated in a plant mixture on a soil highly polluted with zinc, cadmium and lead, *A. vulneraria* in association with *M. metallidurans* increased the biomass of two grass species growing in its vicinity and the total N level in the soil (Frérot et al. 2006). However, no measures of N fixation were made during this study.

The purpose of this work was to test the specificity and efficiency of the symbiotic association *Mesorhizobium metallidurans/Anthyllis vulneraria* in fixing N in a highly contaminated soil from the Mediterranean region. Symbiotic metallicolous and non-metallicolous rhizobial bacteria were isolated from *A. vulneraria* nodules grown *in situ* on metal-contaminated or on neighbour non-contaminated calcareous soils, respectively. The metallicolous and non-metallicolous rhizobia were inoculated in each combination of soil type (metal-contaminated or not) and plant subspecies (metallicolous or not). A greenhouse study was then conducted to evaluate soil-rhizobium-plant impacts on:

(1) Growth responses and survival performances of the metallicolous subspecies of *A. vulneraria* compared with the non-metallicolous species when

grown on an unpolluted soil and on a metal-contaminated soil in association with metallicolous or non-metallicolous strains of *Rhizobium*.
(2) Symbiotic performances including nodulation and N_2-fixing potential of different combinations of *A. vulneraria*-rhizobium associations and soil types.
(3) Heavy metal accumulations in plant shoots, concerning Zn, Pb and Cd, which were the main heavy metals detected in the contaminated soil used.

Materials and methods

Anthyllis *populations*

Seeds of *Anthyllis vulneraria* growing on Zn, Pb and Cd contaminated soil were collected from the Les Avinières mine in the area of Saint-Laurent-le-Minier, 40 km north of Montpellier, France (Frérot et al. 2006). This metallicolous population belongs to the subsp. *carpatica* and will be referred to as Met *Anthyllis*. The population of *A. vulneraria* collected on calcareous non-contaminated soil on the southern border of the Larzac Plateau (France), 25 km SW from the Les Avinières site, belongs to the subsp. *praepropera* and will be referred to as Non-met *Anthyllis*.

Soil characteristics

The soil characteristics of both sites are listed in Table 1. The contaminated soil was collected in the tailing ponds from the Les Avinières mine at Saint-Laurent-le-Minier (hereafter "mine tailings", Escarré et al. 2010). This soil is a human-induced extreme environment, very unfertile with a low organic matter content and a shortage of major plant nutrients. In order to estimate the potential fertility of the contaminated soil, we chose soil from where the Non-met *A. vulneraria* subsp. *praepropera* population grows naturally in the Larzac Plateau as the control. This soil is a calcareous alkaline soil with a low level of P_2O_5 (hereafter "unpolluted soil").

The total metal concentrations were extremely high (total Zn: 161,000 mg kg^{-1}, Pb: 92,700 mg kg^{-1}, Cd: 1,382 mg kg^{-1}) (Frérot et al. 2006). To analyse the extractable metal concentration in the soils, air-dried soil (20 g) was shaken for 30 min with 100 ml of extracting solution (0.5 M ammonium acetate, 0.5 M acetic acid and 0.02 M EDTA at pH 4.65) (Cottenie et al. 1982). The extracts were filtered on folded Schleicher & Schuell 595 1/2 filters (125 mm diameter). Total and extractable metal concentrations were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista MPX).

Bacterial isolation and description of *Mesorhizobium* strains

Nodules were collected from Met and Non-met *Anthyllis* plants growing under field conditions in each of their respective sites. The isolation, identification and multiplication of the symbiont were made following the procedure described by Vidal et al. (2009). The strains STM 2683 and STM 2682 were used as metallicolous (hereafter Met Rh) and non metallicolous *Rhizobium* (hereafter Non-met Rh). The strain STM 2683 was identified as a novel species, *Mesorhizobium metallidurans* sp. *nov.* tolerant to high Zn and Cd concentrations (Vidal et al. 2009). The strain STM 2682 was closed to *M. metallidurans* (99.8% of 16S rDNA similarity) and showing a very low tolerance to Zn and Cd (See Results). Strain STM 2682 could be designated as *Mesorhizobium* sp. STM 2682 from a different evolutionary lineage (C. Vidal, unpublished data).

Bacterial survival in unpolluted soil and in mine tailings

The two soils were air dried and passed through a 2 mm sieve before transferring 10 g samples into 50 ml centrifuge tubes (Falcon^{TM}). Each tube was supplemented with 3.6 ml of deionized water for the unpolluted soil and 2.8 ml for the mine tailings. The soils were sterilized twice by autoclaving at 120°C for 20 min and were left for 4 weeks after sterilization to limit the possible toxic effect of sterilization.

A loop of 72-h-old Petri dish culture strains Met Rh and Non-met Rh were transferred into 10 ml of fresh liquid medium. After aerobic growth for 72 h at 28°C, the cultures were centrifuged and the cell pellets were re-suspended in 3 ml of 0.85% (W/V) NaCl before adjusting the cell suspensions to a final concentration of about 10^7 CFU (colony forming units) ml^{-1}. Then, 0.5 ml of Met Rh or 0.5 ml of Non-met Rh suspensions were introduced into both soils.

Table 1 Characteristics of the two soils used in the experimental treatments

Soil type	pH	OM[a] (%)	Clay (%)	N (%)	P_2O_5 (g kg^{-1})	CEC (cmol kg^{-1})	Zn[b] (mg kg^{-1})	Pb[b] (mg kg^{-1})	Cd[b] (mg kg^{-1})
Unpolluted soil	8.47	2.65	16.2	0.14	0.010	7.6	1.1	2.4	0.2
Mine tailings	7.57	0.18	1.7	0.013	0.008	1.0	35,000	25,300	16.5

[a] organic matter

[b] acetate-EDTA extractable

At inoculation time (T0), and 3 days (T3), 7 days (T7), 15 days (T15), 22 days (T30) and 45 days (T45) after inoculating the soils, the CFU g^{-1} soil values were determined on YEM agar by shaking the incubated soil samples in 100 ml of sterile water where 5 g of glass beads had been added.

Seed treatments, germination and transplanting

The Met and Non-met *A. vulneraria* seeds were surface sterilized and germinated as described above. The seedlings were transferred onto a sterile organic substrate at pH 7.5 and placed for 1 month in a greenhouse with 24°C temperature and 60% humidity under artificial light at a photon flux density of 130 μmol m^{-2} s^{-1}. The plantlets were then individually transferred into containers filled with 450 g of non-sterilized soil (mine tailings or unpolluted soil) and inoculated with the rhizobial inoculant (10^9 CFU $plantlet^{-1}$).

Greenhouse experimental design

The experiment consisted of a complete block design composed of four blocks with three factors tested: soil type (unpolluted soil vs. mine tailings); *Anthyllis* subspecies (Met vs. Non-met) and *Rhizobium* treatment (metallicolous, non-metallicolous *Rhizobium* and non-inoculated control), giving a total of 12 treatments. Fifteen plant replicates per treatment were tested. Plants of the metal-tolerant species *Thlaspi caerulescens* were also introduced to the experiment as a non-N_2-fixing control plant to estimate the proportion of total fixed N derived from the atmosphere using the ^{15}N labelling method. As for *A. vulneraria* seeds, *T. caerulescens* seeds were collected from the Les Avinières mine tailings in the area of Saint-Laurent-le-Minier (Escarré et al. 2000). The plants were randomly distributed in a glasshouse under a natural day: night regime and the temperature (25°C/18°C light/dark) and humidity (70–80%/60–65% light/dark) were controlled. The plants were supplied weekly from day 0 to day 66 after transplanting with a mineral solution containing 1 mM $Ca(NO_3)_2$ (Bertrand et al. 2000) and daily with distilled water after day 66. The plants were harvested for measurements 210 days after transplanting.

Proportion of total N uptake derived from the atmosphere

Twenty milligrammes of N per kilogramme of soil containing 10 atom % ^{15}N excess NH_4NO_3 was applied in solution to the inoculated and the control pots after 66 days of growing under glasshouse conditions. The plants were then watered daily with distilled water until the end of the experiment. The proportion of nitrogen derived from the atmosphere (N_{dfa}) in the test plant was calculated using the following equation:

$$\%\ Ndfa = \left[1 - \frac{\text{atom \% 15N excess in fixing plant}}{\text{atom \% 15N excess in reference plant}}\right] \times 100$$

This formula was applied to the fixing and reference plants growing on the same soil type (unpolluted or mine tailings) and the mean atom % ^{15}N excess in the reference plant *T. caerulescens* was calculated from five replicates for each soil type. The atom % ^{15}N excess and the total nitrogen content were measured by a Thermo Electron mass spectrophotometer (Delta Plus Advantage type, Bremen, Germany) coupled to a Eurovector elemental analyser (Euro EA type, Milan, Italy).

Plant and nodule biomass, metal contents

The number of nodules was determined 80 and 210 days after transplanting on five and eight plants per treatment, respectively. Plants were removed and oven dried at 60°C for 3 days before determining the biomass of the

nodules and aerial parts. Plants that did not grow normally (i.e. in treatments 6, 7, 8 involving non-metallicolous plants growing on the metal-contaminated soil) were collected before the end of the experiment when they died. The total metal contents (Zn, Pb and Cd) in the plants were determined from five replicates per treatment. Subsamples of aerial parts were washed in deionized water, oven dried at 60°C for 3 days, reduced to a fine powder before mineralization in a mixture of nitric and perchloric acid with a Tecator™ digestor, and then analysed by ICP-OES as for the soil analysis described above.

Statistical analyses

Data were analysed by a three-way ANOVA, performed with SAS (SAS 1985) with type III sums of squares because of the occurrence of missing values. Three factors were studied in this experiment: soil type, plant subspecies and *Rhizobium* inoculation. Since all plants of the Non-met *A. vulneraria* died during the first month of the experiment when grown on soil from mine tailings, the corresponding values were zeros and data were not normally distributed despite a logarithmic transformation. Even though ANOVA is considered to be robust for most types of non-normality (Underwood 1981), we re-analysed the data with a one-way ANOVA after logarithmic transformation to normalize the values, with each combination of the three factors as an independent term of the model (nine modalities) and excluding the three modalities of Non-met *A. vulneraria*×contaminated soil×*Mesorhizobium spp.* (or control). After the ANOVA calculations, we utilized *a posteriori* contrasts to test the differences between treatments, especially those that showed significant differences in the three-way ANOVA. Comparisons of means were performed with a Tukey test (SAS 1985).

Results

Bacterial isolation and survival in sterilized soils

Metallicolous (STM 2683, Met Rh) and non-metallicolous *Mesorhizobium* strains (STM 2682, Non-Met Rh) were isolated from root nodules of metallicolous (Met) and non-metallicolous (Non-met) *Anthyllis vulneraria*, respectively. The two *Mesorhizobium* strains exhibited similar kinetics throughout the 45 days after their introduction into the non-contaminated soil (Fig. 1). The population densities increased from about 10^5 to 10^7-10^8 CFU before equilibrium or a slight decrease. On the mine tailings, the two strains exhibited different behaviours: the non-metallicolous *Mesorhizobium* strain completely disappeared from the mine tailings after 3 days of incubation, whereas the metallicolous strain reached a constant level at about 10^4 cells g^{-1} (Fig. 1). Similar results were observed when the metal tolerance of the both *Mesorhizobium* strains was tested on plates containing a medium supplemented with high contents of Zn (unpublished data).

Effects of the plant subspecies and rhizobial inoculation on plant growth, nodulation, N_2 fixation and metal accumulation when plants were grown on the unpolluted soil

Plant growth The results of the three-way ANOVA showed important differences between the soils and an interaction between soil×subsp. (Table 2) because Non-met *Anthyllis* did not grow in the mine tailings. In the unpolluted soil, regardless of the inoculation treatments, the Non-met *Anthyllis* exhibited a mean shoot dry mass (4.78 g $plant^{-1}$, Table 3) significantly

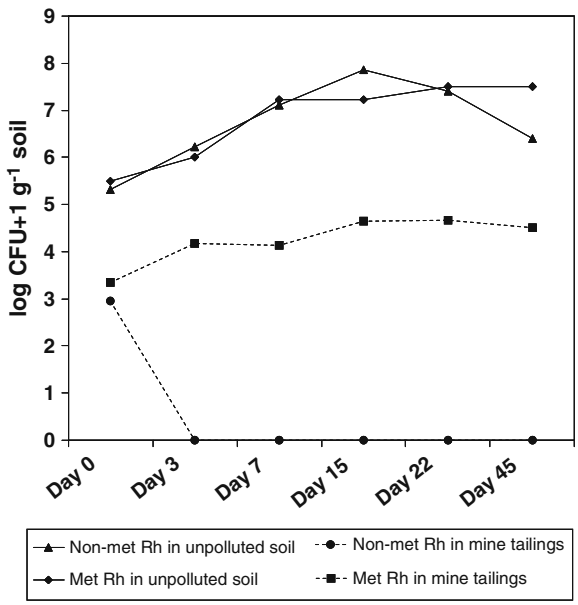

Fig. 1 Survival kinetics of the metallicolous rhizobium (*Met Rh*) and non-metallicolous rhizobium (*Non-met Rh*) strains in unpolluted soil and in mine tailings over 45 days

higher by 74% ($P<0.001$) than that of the Met *Anthyllis* (2.75 g plant^{-1}) (Table 2, contrast between subspecies and unpolluted soil). The inoculation conditions had no significant effects on legume biomass when both subspecies were analysed together (Table 2, non-significant contrast between Rh vs. control). Yet, within the Met *Anthyllis* data alone, plants inoculated with the Met-Rh strain showed a significantly lower shoot dry mass than that of plants inoculated with the Non met-Rh strain (Table 3).

Nodulation The three-way ANOVA calculations showed a complex interaction between soil × subsp. × Rh for both nodule number and biomass (Table 2). The Non-met *Anthyllis* (Table 3) harboured a mean number of nodules (520 plant^{-1} at day 210) and a mean nodule dry mass (129 mg plant^{-1})on its roots that were respectively 160% and 74% higher than those of the Met *Anthyllis* (200 nodules plant^{-1} and 74 mg of nodules plant^{-1}) (Table 2, significant contrast bwteen subspecies in unpolluted soil; $P<0.001$). However, the inoculation treatments had no overall effect at day 210 (non-significant contrast between Rh in unpolluted soil; $P>0.05$) (Table 2). Furthermore, regarding nodule numbers, the rhizobial strains had a contrasting impact depending on the legume subspecies (Table 3). Indeed, compared to the Non-met Rh strain, the addition of the Met-Rh triggered twice as many nodules in the Non-met *Anthyllis* whereas it only initiated half the number of nodules in the Met *Anthyllis* after 210 days of growth (Table 3). These behaviours were already detectable at day 80, except for the non-inoculated Met *Anthyllis* individuals where nodulation started poorly with only a few nodules per plant. The effects of rhizobium addition on the nodule numbers counted at day 210 almost disappeared for the nodule biomass data collected on the Non-met *Anthyllis*, whereas they were still measurable on the Met subspecies (Table 3). This means that Met-Rh produced higher numbers of nodules on Non-met *Anthyllis* but the nodules were smaller, while on the Met subspecies a lower number of nodules and nodule biomass was generated.

Nitrogen fixation There was a complex three-way interaction for the nitrogen measurements i.e. total shoot N and % N_{dfa} (Table 2). For all inoculation treatments combined, the Non-met subspecies had higher values of total N (87 mg plant^{-1}), % N_{dfa}(75%) than the Met subspecies(54 mg plant^{-1} of total N; 62% N_{dfa}, respectively) (Table 3). This lower N fixation of the symbiotic coupling between Met Rh×Met *Anthyllis* is in agreement with its lower nodulation ability (nodule number and total biomass). An inoculation impact was only demonstrated for the % N_{dfa} data (Table 3) in which Met Rh fixed less nitrogen (contrast between Rh in unpolluted soil). Although the inoculation conditions had no significant effect on the total N (non-significant contrast between rhizobia), an inoculation×plant subspecies interaction was noticed on % N_{dfa} (significant contrast between Rh in unpolluted soil; Table 3). The Met *Anthyllis* associated with Met Rh contained about 50% less total N compared to the non-inoculated conditions or the Non-met Rh addition in unpolluted soil, whereas the non-inoculated Non-met *Anthyllis* had 30% less total nitrogen than when inoculated (Table 3).

Metal accumulation For all inoculation treatments combined, the Non-met subspecies accumulated a significantly higher concentration of Zn (mean of 162 μg g-1 of dry shoot; Table 3)corresponding to 45% more than in the Met *Anthyllis* (112 μg g^{-1}) (Table 2, significant contrast between subspecies in unpolluted soil). No inoculation effect was evident but there was a three-way interaction ($P<0.05$) on Zn accumulation. Non-inoculated Non-met plants concentrated significantly more Zn than plants inoculated with Met Rh, whereas non-inoculated Met individuals tended to accumulate less Zn compared with plants previously inoculated with Non-met Rh rhizobia. In contrast, the Pb and Cd contents were similar in the shoots of plants of both *Anthyllis* subspecies (mean of 29 mg Pb and 1.8 mg Cd g^{-1} of plant).

Effects of soil type and rhizobial inoculation on plant growth, nodulation, nitrogen fixation and metal accumulation in the metallicolous subspecies

Plant growth The Met subspecies grew significantly better (Table 3) in unpolluted soil than in metal-rich soil (Table 2, significant contrast between soils for Met *Anthyllis*). Its mean shoot dry mass (2.75 g plant^{-1}) was twice as high as that of plants grown on mine tailings (1.31 g plant^{-1}). In contrast, the Non-met plants only grew on the unpolluted soil and completely degenerated on mine tailings (Table 3). The shoot dry mass of

Table 2 Significance levels obtained from three-way ANOVA calculations testing the effects of soil type, *Anthyllis vulneraria* subspecies and rhizobium inoculation factors on plant growth, nodulation and nitrogen fixation (N_{dfa}) data and metal concentrations of shoots in metallicolous and non-metallicolous *A. vulneraria* subspecies, + $P<0.10$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Variable	DF	Nodule number/ plant Day 80	Nodule number/ plant Day 210	Nodule dry mass (mg plant^{-1}) Day 210	Shoot dry mass (g plant^{-1})	Shoot N (mg p lant^{-1})	N_{dfa} (%)	Zn concentration (μg g^{-1})	Pb concentration (μg g^{-1})	Cd concentration (μg g^{-1})
		Three-way ANOVA								
Soil	1	F=39.7***	F=100.1***	F=100.6***	F=167.0***	F=254.9***	F=168.93***	F=182.5***	F=218.3***	F=52.4***
Subspecies	1	3.9+	12.7***	1.3	2.5	0.3	190.11***	199.9***	254.8***	62.7***
Soil×Subspecies	1	13.2***	45.6***	31.0***	47.9***	75.5***	374.53***	208.0***	255.0***	67.3***
Rhizobium	2	13.4***	2.9+	0.3	0.3	2.4	2.25	3.0+	1.5	1.1
Soil×Rhizobium	2	6.6**	0.4	1.3	0.4	3.9*	4.25*	2.6+	1.8	0.8
Subspecies×Rhizobium	2	6.0**	8.3***	0.9	1.1	5.7**	1.98	2.3	0.9	0.8
Soil×Subspecies×Rhizobium	2	14.1***	14.8***	5.6**	1.3	7.1**	5.06**	3.4*	2.6+	1.3
Univariate ANOVA										
	8	F=9.9***	F=10.1***	F=7.5***	F=18.3***	F=229.8***	F=687.0***	F=159.6***	F=33.1***	F=40.2***
A posteriori contrasts										
b/n (*) Soils	1	14.9***	35.4***	30.5***	53.8***	70.6***	6.4*	1226.1***	249.5***	314.1***
subspecies Met b/n soils	1	1.7	3.9*	7.4**	13.8***	36.5***	10.1**	1019.9***	196.8***	254.2***
b/n Subspecies on unpolluted soil	1	16.5***	39.9***	16.9***	27.7***	6.1*	3.9+	8.28**	0.5	2.6
Rhizobium vs. Non-inoculated	1	27.7***	0.0	0.2	0.1	7.56**	1.3	4.15*	0.6	0.2
b/n Rh on unpolluted soil	1	0	4.1*	0.2	0.8	0.1	10.2**	3.1+	1.5	0.2
Met Rh vs. Non-inoculated on mine tailings	1	17.8***	0.5	1.4	0.1	0.1	0.2	1.7	0.1	0.6
Met Rh vs. Non-inoculated on unpolluted soil	1	15.5***	1.3	0.9	0.5	8.0**	0.3	3.79+	1.2	0.1
Non-met Rh vs. control on unpolluted soil	1	15.1***	0.8	0.3	0.1	8.6**	6.8*	0.1	0.1	0.1

(*) b/n and vs. are abbreviations for between and versus, respectively

Table 3 Effects of soil type, plant subspecies and inoculation on nodulation, plant growth, nitrogen fixation (N_{dfa}) and metal accumulation in *Anthyllis vulneraria* grown for 8 months in a greenhouse+$P<0.10$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Soil type and plant origin [a]	*Rhizobium* inoculant [b]	Nodule number/plant Day 80	Nodule number/plant Day 210	Nodule dry mass (mg plant^{-1}) Day 210	Shoot dry mass (g plant^{-1})	Shoot N (mg plant^{-1})	N_{dfa} (%)	Zn concentration (μg g^{-1})	Pb concentration (μg g^{-1})	Cd concentration (μg g^{-1})
Unpolluted soil										
Non-met *A. vulneraria*	Non-met Rh	52±17 b [c]	335±56 b	107±11 bc	4.56±0.47 a	95.1±9.1 a	72.3±6.5 a	127±13 c	19±7 c	1.7±0.2 c
	Met Rh	162±39 a	794±98 a	147±19 a	5.01±0.48 a	97.5±8.8 a	75.3±7.7 ab	115±15 c	25±9 c	1.7±0.3 c
	Non-inoculated	19±7 bc	433±117 b	135±29 ab	4.79±1.11 a	67.0±7.3 b	76.1±5.8 ab	245±43 b	43±10 c	2.8±0.9 c
Met *A. vulneraria*	Non-met Rh	76±30 b	265±63 bc	96±21 bcd	3.33±0.26 b	70.3±9.9 b	67.2±10.0 b	146±17 c	48±15 c	1.8±0.4 c
	Met Rh	24±12 bc	58±27 d	41±16 e	2.00±0.23 cd	31.5±7.7 c	46.1±20.7 c	103±16 c	25±14 c	1.2±0.4 c
	Non-inoculated	2±1 c	278±57 bc	85±17 cd	2.92±0.38 bc	60.9±6.1 b	71.8±7.5 ab	86±13 c	14±3 c	1.3±0.6 c
Mine tailings										
Non-met *A. vulneraria*	Non-met Rh	–	–[d]	–	0.055±0.019 [e]	–	–	–	–	–
	Met Rh	–	–	–	0.040±0.009[e]	–	–	–	–	–
	Non-inoculated	–	–	–	0.046±0.014[e]	–	–	–	–	–
Met *A. vulneraria*	Non-met Rh	3±2 c	46±13 d	20±3 e	1.25±0.14 d	25.6±4.3 c	71.0±13.7 ab	4,294±425 b	669±76 b	29.7±2.6 b
	Met Rh	34±7 bc	156±15 cd	59±10 de	1.33±0.11 d	31.4±1.3 c	80.5±4.8 a	4,704±491 b	775±53 ab	30.6±5.5 b
	Non-inoculated	1±1 c	95±13 d	31±6 e	1.34±0.16 d	29.0±3.7 c	77.6±4.6 ab	6,229±813 a	884±110 a	43±11.2 a

[a] Non-met *A. vulneraria*: *A. vulneraria* subspecies from unpolluted soil; Met *A. vulneraria*: *A. vulneraria* subspecies from mine tailings

[b] Non-met Rh: non-metallicolous rhizobium strain; Met Rh: metallicolous rhizobium strain

[c] Least square means tests were conducted after a three-way ANOVA: same letters indicate an absence of significance ($P<0.05$)

[d] not measured because the plants died before the end of the experiment

[e] biomass of plants dead before day 80

the Met subspecies grown on unpolluted soil was greater (× 1.4) when inoculated with Non-met Rh than when inoculated with Met Rh, whereas the shoot dry mass remained similar whatever the inoculation conditions when growing on mine tailings (Table 3).

Nodulation The soil type had a highly significant effect ($P<0.01$) on both nodule number and nodule dry mass in the Met subspecies, whereas the inoculation factor had no effect (Table 2). For all inoculation treatments combined, the Met plants grown on unpolluted soil had mean nodule numbers and a nodule dry mass twice as high as those of plants grown on mine tailings (Table 3). Moreover, the three-way interaction was significant ($P<0.01$) (Table 2). The Met *Anthyllis* inoculated with the Met Rh strain showed the lowest infectivity in terms of nodule number and biomass when grown on the unpolluted soil (Table 3), whereas they had the highest infectivity on the toxic soil (Table 2, significant contrast between soils for Met *Anthyllis*).

Nitrogen fixation Soil type was a highly significant factor ($P<0.001$) on all N_2-fixation data analysed in the Met subspecies (Table 2, significant contrasts between soils and Met *Anthyllis* for total N and % N_{dfa}). For all inoculation treatments taken together, the Met plants grown on unpolluted soil had values of total N (54 mg N) and total N fixed (35 mg N) about twice as high as the values of total N for plants grown on mine tailings (Table 3). In contrast, the proportion of fixed N (% N_{dfa}) was higher in plants grown on mine tailings (76%) than in plants grown on unpolluted soil(61%) (Table 3). On the other hand, the inoculation conditions had only a slightly significant effect on total N, while the three-way interaction between the inoculation, soil type factors and Rh had significant impacts on all N_2-fixation data (Table 2). In the unpolluted soil, the Met plants inoculated with the Met Rh strain fixed significantly less N (46%) and contained less total fixed N (15 mg N), whereas in the mine tailings Met plants inoculated with Met Rh reached equivalent % N_{dfa} (76%) and total N (mean of 22 mg fixed N) values to the Met plants not inoculated or inoculated with Non-met Rh (Table 2).

Metal accumulation The concentrations of Zn, Pb and Cd contained in Met *Anthyllis* leaves differed significantly ($P<0.001$) between the two soil types (Table 2, significant contrast between soils for Met *Anthyllis*). For all inoculation treatments combined, the Met plants in mine tailings accumulated mean concentrations of Zn, Pb and Cd far higher than those measured when the Met plants grew on the unpolluted soil (5,076 μg g^{-1} vs. 112 μg g^{-1}, 776 μg g^{-1} vs. 29 μg g^{-1} and 34.4 μg g^{-1} vs. 1.5 μg g^{-1}, respectively) (Table 3). Plant metal contents were significantly higher in non-inoculated Met plants on mine tailings (Table 3).

Discussion

Rhizobium survival in heavy metal-contaminated soil

The mine tailings used in the experiments are among the most contaminated in terms of zinc, cadmium and lead from the mine sites inventoried in Europe (Escarré et al. 2010). The disappearance of the non-metallicolous rhizobium population within 3 days of incubation in the mine tailings showed the total inability of the strain originating from a non-contaminated soil to survive in mine tailings. In contrast, the Met Rh strain originating from a metal-rich soil remained at a high population level, even after 45 days in the mine tailings. The initial difference observed at the beginning of the experiment between rhizobial populations in the two soils was not due to a heavy metal effect but rather to the difficulty of extracting all the bacteria from the soil and to the recovery rates that are soil-dependent (Crozat and Cleyet-Marel 1984). Compared to Cd, Zn has been found to be the most toxic metal to free-living rhizobia in sludge-amended soils in the absence of the host plant (Chaudri et al. 1993; Broos et al. 2005), and generally the rhizobium severely declines between 230 and 880 mg Zn kg^{-1}, depending on the soils. Thus, an inhibition of nitrogen fixation has been observed at moderate zinc concentrations in soils, for example 366 mg Zn kg^{-1} for broad bean (*Vicea faba*) and pea (*Pisum sativum*) inoculated with their symbiont *Rhizobium leguminosarum* biovar. *vicea* (Obbard and Jones 2001) and 614 mg Zn kg^{-1} for non-inoculated white clover (*Trifolium repens*) (Broos et al. 2004). Nodulation of broad bean and pea totally failed at 664 mg Zn kg^{-1} soil (Obbard and Jones 2001). Our results clearly indicate that the Met Rh

strain isolated from a metallicolous *A. vulneraria* nodule is a metal-tolerant bacterium. Moreover, the metal concentration in the mine spoils at Les Avinières (Table 1) is far higher than that of sludge-amended soils where the effects of metal toxicity on symbiotic nitrogen fixation have previously been studied (Obbard and Jones 2001; Broos et al. 2004) and consequently Met Rh exhibited a remarkably high Zn tolerance level.

The survival of a healthy population of the micro-symbiont appears to be the most critical factor influencing N fixation (Broos et al. 2004, 2005). In other studies, Castro et al. (1997) showed that only 15% of a *Rhizobium leguminosarum* cv. *trifolii* population, isolated from an industrial area polluted for nearly 40 years and containing various metals such as Zn (117 mg kg^{-1}) and Ni (375 mg kg^{-1}), was effective against 94% in an unpolluted control. Lakzian et al. (2002) observed an increase in the number of plasmid profile groups at a moderate concentration of Zn and a strong reduction when the metal concentration exceeded 300 mg Zn kg^{1}. The formation of ineffective nodules was also observed on white clover grown in soil polluted by applications of sewage sludge (McGrath et al. 1988; Chaudhary et al. 2004). Symbiotically effective *Sinorhizobium meliloti* strains tolerant to As, Cu and Pb were found in soils contaminated after the toxic spill at the Aznalcollar pyrite mine (Carrasco et al. 2005). To a large extent, only one population of white clover (*T. repens*) was identified to have conserved a potential for nitrogen fixation on soil highly contaminated with Zn i.e. containing up to 30,000 mg Pb kg^{-1} soil and 20,000 mg Zn kg^{-1} soil (Rother et al. 1982).

These results highlight the extraordinary metal tolerance of this novel species *Mesorhizobium metallidurans* (Vidal et al. 2009) as a legume symbiotic bacteria with saprophytic activity in soil contaminated with zinc, lead and cadmium which is comparable to *Arthrobacter* spp. isolated from mine tailings and *Cupriavidus* (formerly *Wautersia/Ralstonia) metallidurans*, a model metal-tolerant bacterium (Mergeay et al. 2003; Vaneechoutte et al. 2004).

Biomass production of *A. vulneraria* in unpolluted and in metal-contaminated soils

Data on plant biomass production indicate that the growth of the non-metallicolous *A. vulneraria* is completely inhibited on mine tailings regardless of the inoculation management, and the plants died a few weeks after the beginning of the experiment. This result was also found by Frérot et al. 2006 which showed a significant lower tolerance to Zn of the non met subspecies compared to plants from the mine site of Les Avinières using the sequential method of Schat and Ten Bokum (1992). On unpolluted soil, the non-metallicolous *A. vulneraria* developed well and had the higher biomass. The metallicolous *A. vulneraria* grows on both mine tailings and unpolluted soils but the growth on the former is reduced by a factor of 2.5. An interesting observation was that the shoot dry mass of Met *Anthyllis* was significantly reduced in unpolluted soil when inoculated with Met Rh, the metallicolous inoculant rhizobium. This result suggests that the association between the Met *A. vulneraria* with the metallicolous rhizobium is not adapted to unpolluted soil and has a 'need' for heavy metals. Such higher performances of non-metallicolous plant populations on unpolluted soils were reported by Mathys (1977), who found a stimulating effect of Zn on growth only in metallicolous populations, and by Escarré et al. (2000) who reported a better adaptation of non-metallicolous *Thlaspi caerulescens* populations than metallicolous ones in unpolluted soils. This observation suggests that Met *A. vulneraria* is adapted to metal-rich soils and the requirement for Zn is not achieved in unpolluted soil when inoculated with Met Rh. When it grows on soil rich with zinc, lead and cadmium, the aerial parts of Met *A. vulneraria* accumulated the metal at high concentrations: 4,000 μg g^{-1} for Zn, 600 μg g^{-1} for Pb and 30 μg g^{-1} for Cd. This represents 30–70 times more Zn that when cultivated in unpolluted soil. Nevertheless, the Zn content in the shoot of the metalliferous *A. vulneraria* remains below the hyper-accumulation threshold defined by Baker and Walker (1990). Other legumes such *Ononis natrix*, or small forb legumes, were identified on contaminated soils in Moroccan mines (Boularbah et al. 2006) and after a toxic spill in Spain (Carrasco et al. 2005), yet the differences in natural legume vegetation could be due to Zn concentrations that were at least 100-fold lower than those of the St-Laurent-le-Minier mining area. The growth of the metallicollous *A. vulneraria* subspecies on extremely metal-contaminated soils is highly specific in terms of heavy metal concentrations.

Symbiotic properties of the *A. vulneraria*-rhizobium association

The low number of nodules recorded on the control plants 80 days after transplantation and inoculation indicate that very few rhizobia able to form nodules in Met *Anthyllis* were naturally present at the beginning of the experiment in both unpolluted and in metal-contaminated soil. In mine tailings, inoculation of metallicolous *A. vulneraria* with the Non-met Rh strain did not increase the number of nodules at day 80, and this result is in agreement with the inability of the Non-met Rh strain to survive in the metal-contaminated soil. In unpolluted soil, the non-metallicolous *A. vulneraria* showed a spontaneous nodulation and inoculation with the Non-met Rh strain slightly increasing nodulation, whereas a high number of nodules was observed when the same plant subspecies was inoculated with Met Rh. An unexpected result was the high number of nodules observed in the control plants at day 210. This end result could be explained by the long duration of the experiment or the low N fertilization application during the first 80 days which allowed initial plant growth and thus permitted rhizosphere multiplication of the native rhizobial populations. In unpolluted soil, the two plant subspecies had more nodules when they were inoculated with the non-legitimate bacterial strain than with their genuine rhizobium (Table 2). This observation draws attention to the complexity of the nodulation regulation that is integrated into the overall regulatory circuits involving both external and internal factors to the plant host (Kosslak and Ben Bohlool 1984; Delves et al. 1986) and sophisticated signalling mechanisms contributing to the autoregulation of nodule numbers (Krussell et al. 2002; Loh and Stacey 2003).

Despite the clear differences in nodule numbers for the different *Rhizobium* treatments (nos. 1 and 2) applied to the Non-met *Anthyllis*, no difference was observed in nodule biomass, nor in % N_{dfa} since 72–75% of the total nitrogen was derived from biological nitrogen fixation. This implies that in unpolluted soil, each nodule initiated by the metallicolous strain on the Non-met *Anthyllis* subspecies was less efficient in nitrogen fixation and that the plant compensated for the poor nitrogen efficiency by increasing the number of nodules. Numerous nodules were observed on the Met *Anthyllis* inoculated with the metallicolous strain in the mine tailings, resulting in 80% of total nitrogen being derived from biological N_2 fixation. In contrast, when grown on unpolluted soil, this plant-bacterium association had a lower number of nodules, a reduced nodule dry mass and a low % N_{dfa} (less than 50% of the N was derived from the atmosphere). Therefore, the significant reduction in shoot dry mass observed and discussed previously was probably a consequence of the lower nitrogen potential. Our observations on N_2 fixation with *A. vulneraria* established in a metal-contaminated soil are in contrast with a survey of symbiotic N fixation on white clover (*Trifolium repens*) grown on soils contaminated with heavy metals after sludge amendment (Obbard and Jones 2001; Broos et al. 2004). In these studies, the total Zn concentrations able to reduce the amount of N_2 fixation of white clover from 40% to 15-16% were about 297–542 mg kg^{-1} of dry soil (Obbard and Jones 2001), whereas the total N derived from biological N_2 fixation in our experiment reached 80% for *A. vulneraria* grown in a substrate containing 160,000 mg kg^{-1} of total Zn of dry soil. Moreover, the mine tailings used in our experiment contained high levels of Pb and Cd, two other heavy metals which are highly toxic to plants and bacteria. Mine soils generally have low organic matter contents and N_2-fixing plants have facilitating effects on the installation of other plant species (Harper 1977). Frérot et al. (2006) recently showed that in a mixture with non-legume species, the metallicolous *A. vulneraria* increased the biomass production of the plant community when grown on highly metal-contaminated soil.

Conclusion

The present work highlights the capacity of a rare *A. vulneraria* population to grow in a mining soil strongly contaminated with heavy metals (16% Zn, 9.2% Pb and 1.3% Cd). This specific legume is able to establish a symbiosis with a highly metal-tolerant rhizobium strain generating an efficient symbiosis able to fix 80% of its total nitrogen from atmospheric N_2. Such a hypertolerant and functional symbiosis can promote vegetation cover, stabilizing metal-enriched soils, and therefore it could be an attractive tool for phytostabilization, particularly in the Mediterranean region where facilitation processes during the first development stages are very important (Sans et al. 1998; Whiting et al. 2004; Frérot et al. 2006).

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