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Specific interactions between local metallicolous plants improve the phytostabilization of mine soils

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Abstract

At present, no efficient technique is available for cleaning up soils which are highly polluted by heavy metals. Limiting the movement of pollutants out of the contaminated area by creating a dense and persistent plant cover appears to be the more reasonable approach. In this context, phytostabilization is a technique that uses metallicolous plants to revegetate highly polluted soils.This paper presents the results of an experiment performed *in situ* using metallicolous ecotypes of four plant species native to the Mediterranean French region, and grown in different combinations at a polluted site over two years. The soils were highly polluted with zinc, cadmium and lead. The aim was to find the best species mixture in terms of cover, biomass and duration. The four species used were the biennial legume *Anthyllis vulneraria*, two perennial grasses, *Festuca arvernensis* and *Koeleria vallesiana*, and the perennial forb *Armeria arenaria*. Mixtures which included *A. vulneraria*, and especially when in combination with *F. arvernensis*, showed the highest values of cover and biomass. After flowering, the biennial individuals of *A. vulneraria* disappeared but subsequent germination and survival of seedlings occurred abundantly under the two grasses. Mixtures with *A. arenaria* showed the lowest values of cover and biomass. Soil nitrogen increased in the plots with *A. vulneraria* as well as the concentration of essential nutrients (N P K) in the aerial parts of the two grasses. In contrast, the concentration of metals (Zn Pb Cd) decreased in the aboveground biomass of the latter in the same plots.These results show that reciprocal facilitation effects can act in heavy metal polluted environments, and that phytostabilization efforts in the Mediterranean region can be improved by using mixtures including local metallicolous legume and grass species.

Introduction

Over the past century, mining industry and urban activities were responsible for extensive heavy metal pollution (Bradshaw et al., 1965). Such polluted areas present a risk for environment and human health, in particular mining waste sites, whose soils contain high amounts of Zn, Cd and Pb (Ernst, 1990). Non-essential metallic elements such as Pb and Cd are toxic at low concentrations, whereas essential ones such as Zn, Fe or Ni, become toxic at excessive concentrations (Borovik, 1990). Remediation methods generally involve stabilising the soil with material such as cement or excavation and land filling of the

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contaminated soil (Berti and Cunningham, 2000). These techniques are destructive and expensive (Chaney et al., 1997). For this reason, the use of heavy metal hyperaccumulating plant species that are able to accumulate and tolerate exceptionally high amounts of heavy metals in their aerial tissues (Reeves and Brooks, 1983) to clean up the soils, i.e. phytoextraction, is now largely considered to be a feasible method (Salt et al., 1995, 1998).

However, in highly polluted areas, where the removal of metals by phytoextraction using hyperaccumulating plants is not efficient due to the slowness of the process (Ernst, 1996), the most suitable method is phytostabilization (Arthur et al., 2005; Salt et al., 1995) This consists in using metallicolous plants, i.e. from metalliferous soils, to establish a persistent plant cover preventing pollution from spreading by erosion, water percolation, leaching and from toxic dust dispersal by wind (particularly in the case of fine-grained material in tailing ponds) (Vangronsveld et al., 1995, 1996). Plant species that have evolved adaptive mechanisms, in particular metal tolerance, to thrive on the metalliferous soils, are called metallophytes (Antonovics et al., 1971). Phytostabilization also involve the use of soil amendments to promote the formation of insoluble metal complexes that reduce their biological availability and plant uptake, thus preventing the metals from entering the food chain (Adriano et al., 2004; Berti and Cunningham, 2000; Cunningham et al., 1995). In addition, as the plants should help to reduce human or animal access to contaminants, they must be metal-tolerant but also non-accumulating. Ideally, metallicolous plants should also be native to the local flora of the mining area in question (Remon et al., 2005), grow quickly and have dense root and shoot systems (Berti and Cunningham, 2000). In the Mediterranean region, native species adapted to water stress are particularly required for a successful remediation because plants have to cope with the long dry summer season in addition to unfertile soil conditions.

Mine soils are generally low in nutrients and organic matter (Bradshaw and Chadwick, 1980). Therefore, the inclusion of nitrogen-fixing metallophytes (such as Fabaceae) in the stabilizing vegetation should improve ecosystem development by naturally increasing the nitrogen content in the soil, and promoting the maintenance of the plant cover (Harris et al., 1996; Jordan et al., 1987; Whiting et al., 2004). In non-contaminated environments, it is well known that legumes have facilitating effects on the installation of other plant species (Harper, 1977). Therefore, it is of great interest for the phytostabilization success to search for an effective mixture of metallophytes, in which some target plants belonging to different functional groups could positively interact with each other.

The objectives of this study are:

(1) To check that metal tolerance has an adaptive base by comparing in controlled hydroponical conditions zinc tolerance of several plant species growing both in old mine sites and non-contaminated sites from the Languedoc-Roussillon Region (South of France), as zinc is the most highly concentrated metal in the mine soils.
(2) To assess growth and survival performances of local metallophytes by cultivating them in various mixtures on a heavily contaminated tailing pond. A particular attention was paid to mixtures including the nitrogen-fixing species *Anthyllis vulneraria* (Fabaceae), whose ability to improve soil fertility, and consequently the yield of the accompanying species, was measured. The final purpose was to find an optimal mixture of species for the installation of a permanent and diversified plant cover.

To the best of our knowledge, this is the first work to use several local metallophyte species to demonstrate that phytostabilization is feasible in highly contaminated tailing sites from the Mediterranean region.

Materials and methods

Species and collection sites

Four non-accumulating species were selected from the most abundant vigorous species growing at the mine sites, whose metal-tolerance, morphology and vegetative reproduction should be suitable for phytostabilization purposes.

Anthyllis vulneraria L. (Fabaceae) (hereafter *Anthyllis*) is an annual, biennial or perennial herb (Couderc, 1975). This very polymorphic species has been divided into many subspecies (Tutin et al., 1993) and is one of the few legume species known for its metal-tolerance (Ernst, 1990; Rascio, 1977). In the Mediterranean region, it occurs on calcareous non-contaminated soils (Bastrenta et al., 1995) and on metalliferous soils, but until now, it has been observed only at "Les Avinières" in the Languedoc-Roussillon mining areas. Its nitrogen-fixing symbiotic activity occurs on non-contaminated soils as well as on contaminated soils (Cleyet Marel, personal communication).

Festuca arvernensis Auquier, Kerguélen and Markgr.-Dann. (Poaceae) (hereafter *Festuca*) is a perennial and cespitose grass belonging to the group of *F. ovina* L. (Tutin et al., 1993), within which metal-tolerant species have been described (Brown and Brinkmann, 1992). In the Mediterranean region, it occurs on calcareous non-contaminated soils, as well as on several mine sites.

Koeleria vallesiana Pers. (Poaceae) (hereafter *Koeleria*) is a perennial grass (Tutin et al., 1993) whose morphology is close to that of *Festuca*. Like the previous species, it occurs on non-contaminated soils and also on calamine soils.

Armeria arenaria (Pers.) Schultes (Plumbaginaceae) (hereafter *Armeria*) is a tufted perennial plant (Tutin et al., 1993). The metal-tolerance of the related species *A. maritima* (Mill.) Willd. has been previously studied by Lefèbvre (1975). *Armeria* can be found on non-contaminated and heavy metal polluted soils.

For the field experiment, seeds from *Festuca* and *Koeleria*, collected from plants growing at a mine abandoned since the Middle Ages (Demange, 1973) and from "Les Avinières" respectively, were sown on calcined clay, and then transplanted to a non-contaminated soil from the Experimental Station of the Centre d'Ecologie Fonctionnelle et Evolutive (CNRS-Montpellier). For *Armeria* and *Anthyllis*, due to a shortage of seeds, seedlings were collected at the "Les Avinières" mine site and then transferred into the same non-contaminated soil. Seedlings of the four species had a similar size when they were transplanted in the field three weeks after germination.

The zinc tolerance of *Anthyllis*, *Festuca* and *Armeria* metallicolous populations was compared with plants of the same species but collected in non-contaminated soil. *Anthyllis* seeds of non-metallicolous individuals were collected in the "Cirque de Navacelles" area, 15 km NW of "Les Avinières." *Festuca* and *Armeria*, seeds of non-metallicolous plants were collected on the "Plateau du Larzac" at 14 and 17 km respectively north of "Les Avinières." For *Koeleria* there were not enough non-metallicolous individuals at the time to perform the zinc tolerance test.

Test of tolerance to zinc in nutritive solution

Zinc tolerance of *Festuca* and *Armeria* was estimated by the method of Wilkins (Wilkins, 1978), which defines the index of tolerance to a metal (It) as the ratio of root growth in a nutritive solution with high zinc concentration to root growth in a non-toxic nutritive solution. Root increment was first measured for 10 days of growth in a non-toxic nutritive solution (Koch et al., 1987) and for a further 10 days in the same solution with the addition of either 1000, 1500, 2000 or 4000 μM of zinc sulphate ($ZnSO_4$ $7H_2O$). As all concentrations showed similar results, only the data for 4000 μM were presented. For the two species, the test compared the metallicolous population of Les Avinières with their respective non-metallicolous populations.

However, since there was no root growth at 1000 μM for non-metallicolous individuals of *Anthyllis*, the tolerance test was switched into the sequential method of Schat and Ten Bookum (1992) for this species.

Seedlings of *Anthyllis* from the populations of Les Avinières (metallicolous) and Navacelles (non-metallicolous), were distributed randomly in black plastic tanks containing 3.5 L of the same nutritive solution as for the two precedent species (Koch et al., 1987). After one month of acclimatization, the solution was supplemented with zinc sulphate and plant roots marked with non-toxic active charcoal (Schat and Ten Bookum, 1992). The surplus charcoal was eliminated by rinsing with distilled water. Charcoal was adsorbed on the roots, which were entirely coloured in black. The new roots appeared as white, indicating that root growth occurred during the period of exposure (one week) to a specific zinc concentration. The plants with black roots (i.e. without new roots) were considered as having reached their tolerance threshold and were withdrawn from the experiment. The roots of the remaining individuals were marked again with the active charcoal and the zinc concentration of the solution was increased by steps of 200 μM of zinc sulphate.

Field experiment

Experimental site, design and species mixtures
The experimental site at the abandoned "Les Avinières" mine, consists of waste rock dumps and tailing ponds. In the latter, total metal concentrations are extremely high (total Zn: 161 000 mg kg^{-1}, Pb: 92 700 mg kg^{-1}, Cd: 1382 mg kg^{-1}). Depth of contaminated soil was two meter and the maximal depth reached by the plant roots was about 25 cm. The soil is very unfertile with no organic matter, a shortage of major plant nutrients (see Results) and a sandy loose soil (50% fine sand), so that contaminated dust can spread by wind dispersal or runoff as heavy rains frequently occur in the Mediterranean region. Such a bare and instable soil prevents seed germination. Therefore, in our experiment, seedlings were used instead of seeds to ensure the rooting phase. To improve soil fertility and facilitate the installation of transplanted seedlings, commercial horse manure (30% organic matter, 1% nitrogen, pH $H_2O = 7.0$) was incorporated into the contaminated soil at about 1.5 kg horse manure per m^2. In a previous experiment (Frérot, 2004), it was showed that horse manure significantly increased the growth of the same set of species. In May 2002, three week old seedlings were transplanted and watered in the experimental site. No additional water was supplied during the experiment.

The experiment was conducted in a three blocks design with 15 plots (60×60 cm) per block. The 15 plots consisted of the four monocultures, the four-species mixture, and the different combinations of two (6 combinations) or three (4 combinations) species. Each plot contained 6×6 individuals. Data were collected only from the 16 inner plants while the 20 others were only considered as border plants. Plots were randomly distributed within each block and were separated from their neighbours by a row 40 cm wide.

Trait measurements
For 24 months, plant survival was checked each month, and plant growth was estimated every two months with the following parameters: leaf number, length of the longest leaf, width of this longest leaf when relevant (i.e. only for *Armeria* and *Anthyllis*), number of inflorescences and length of the longest inflorescence. Previously, these parameters were shown to be significantly correlated with aboveground biomass by performing a multiple linear regression in a sample of plants from the same species, directly harvested in the field ($n = 30$, *Anthyllis* $r^2 = 0.92$, $p < 0.001$; *Armeria* $r^2 = 0.68$, $p < 0.001$; *Festuca* $r^2 = 0.85$, $p < 0.001$; *Koeleria* $r^2 = 0.69$, $p < 0.001$).

As *Anthyllis* is a monocarpic species, it disappeared after flowering in the second year after sowing. Thus, to verify whether the new generation of *Anthyllis* was able to colonize the experimental plots where it was present the year before, the number of seedlings was recorded during the four last months of the experiment.

After 25 months growth, digital pictures of each plot were taken. Cover rates were estimated by superimposing a 100 points grid over each plot picture and by counting the points that corresponded to vegetation. Plant aerial parts were then harvested to measure biomass. Four individuals per species in each plot were taken, i.e. the four central individuals in the monocultures, the two central lines in the two-species mixtures, the three first lines in the three-species mixtures, and all 16 individuals in the four-species mixtures. This allowed the calculation of the mean biomass of each species according to the plot. In a given plot, the total biomass was obtained by multiplying the mean biomass of each present species by the number of individuals of this species in the plot. In each plot, *Anthyllis* seedlings were also harvested when present.

Plant and soil analysis
At the end of the experiment, a bulked sample of 3 subsamples of soil was taken from each plot with *Anthyllis* and without *Anthyllis* at a depth of 10 cm. Mineral elements were extracted with ammonium acetate-EDTA 1 N (pH 4.65) for 30 min (10 g dry soil in 50 ml) (Cottenie et al., 1982). The supernatant was filtered and analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Varian Vista MPX) for the following nine elements: Ca, Mg, P, K, Fe, Mn, Zn and Cd. Plants were harvested, washed in deionised water, oven-dried at 60 °C for

3 days, mineralized in a mixture of nitric and perchloric acid with a Tecator digestor and then analysed by ICP-OES for the same elements as in soil. Total nitrogen (N) was determined with an elemental analyser (Carlo Erba Instruments, model EA 1108, Milan, Italy). Only plants from monocultures and binary mixtures were collected and analysed.

Statistical analyses

Tolerance tests
As no root growth was observable at 1000 μM for non-metallicolous *Anthyllis* individuals, the sequential test of Schat and Ten Bookum (1992) were used for this species, which provides qualitative survival (i.e. tolerance) data at increasing metal concentrations. Tolerance curves for the differences in zinc tolerance between metallicolous and non-metallicolous populations of *Anthyllis* could be analysed as "Survival curves" in which the outcome was time until death. In our case, "death" was the stop of root growth at a particular zinc concentration. Data were thus compared by a logrank test and then fitted by a sigmoidal dose response curve to find the effective concentration of Zn at which 50% of the individuals were non-tolerant (EC50) (Motulsky, 1999).

For the other two species *Festuca* and *Armeria*, comparisons between the tolerance indexes of the two edaphic origins were made by a *t*-test.

Analyses of variance
The data on the estimated biomass for the three species *Armeria, Festuca* and *Koeleria* were log-transformed to improve normalization. The data for *Anthyllis* were not analysed because the biomass values of this biennial species rapidly decreased after the flowering season (see the Results). Differences in estimated biomass between species according to the plot composition and during the 13 measurements were analysed by a repeated two-factor (block and plot) ANOVA (von Ende, 2001), with the GLM procedure of SAS and the "repeated" statement (SAS Institute, 2001). ANOVAs[1] were also performed for each measurement date, and comparisons between means were made by the least-squares means method. The biomass data from the harvest at the 25th month were log-transformed to improve normalization, and comparisons between final biomass produced in plots at the 25th month were performed by a one-way ANOVA using the GLM procedure of SAS and the least-squares means method.

Using the same statistical procedure, the differences between the cover rates, the numbers of inflorescences in each plot at the flowering peak of each species (i.e. in June 2003) and the differences between *Anthyllis* seedling number per plot during the four last months of experiment, were also analysed.

Differences in elemental concentrations among species and treatments were analysed by a two-way ANOVA. Multiple comparisons of mean values were performed using Tukey tests.

Results

Test of zinc tolerance between metallicolous and non-metallicolous populations of Anthyllis, Festuca and Armeria

There were significant differences in tolerance between metallicolous and non-metallicolous plants for the three species tested (Table 1) with metallicolous individuals showing the highest tolerance to zinc, in particular the plants of *Festuca* from Les Avinières. The most striking differences occurred between the two populations of *Armeria*.

Mortality rates in the field

The mortality rate before flowering was high in *Anthyllis* (46%) compared with those of the three other species: only one plant in *Armeria* and *Festuca* (0.006%) and two plants in *Koeleria* (0.01%) were missing at that time. At the end of the experiment, the mortality scores were three plants for *Armeria* and *Koeleria* (0.02%) and five plants for *Festuca* (0.03%) whereas all *Anthyllis* plants had disappeared after flowering as they were biennial.

Plot biomass production and cover rates

Highly significant differences between plots for biomass production appeared during the 2 years of cultivation (Repeated Measures ANOVA, Between Subjects Effects, "plots" effect: df = 13,

Table 1. Test of tolerance between metallicolous and non-metallicolous individuals of *Anthyllis vulneraria* by the sequential method of Schat and Ten Bokum (1992), and of *Festuca arvernensis* and *Armeria arenaria* by the method of Wilkins (1978). See text for additional explanations. EC50: concentration of Zn in which 50% of the individuals were non tolerant. Index of tolerance: the ratio of root length in nutritive solution with a high zinc concentration (4000 μM of Zn) to root length in a non-toxic nutritive solution

Species	Origin	*N*	EC50mean (SE)	Log rank test
Anthyllis vulneraria	Metallicolous	16	1849 (50)	$\chi^2 = 20.8$***
	Non-metallicolous	16	1180 (106)	df = 1
			Index of tolerance	*t* test
Festuca arvernensis	Metallicolous	7	0.81 (0.07)	3.22**
	Non-metallicolous	7	0.36 (0.12)	
Armeria arenaria	Metallicolous	8	0.66 (0.05)	10.8***
	Non-metallicolous	8	0.07(0.02)	

** $p < 0.01$; *** $p < 0.001$.

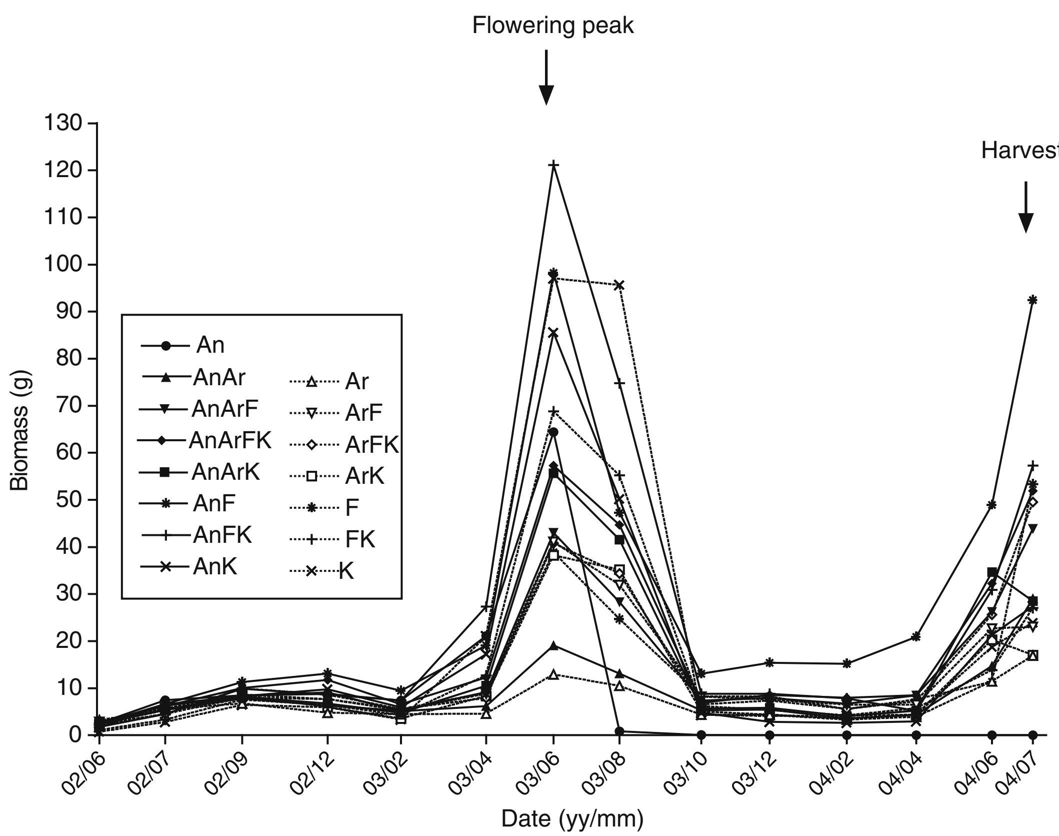

Figure 1. Means of estimated aerial biomass in 13 measurements for the 15 plots of different species mixtures. An: *Anthyllis vulneraria*; Ar: *Armeria arenaria*; F: *Festuca arvernensis*; K: *Koeleria vallesiana*. Connecting lines in bold represent plots where *Anthyllis vulneraria* was initially present.

26, $F = 7.00$, $p < 0.0001$) particularly at the flowering phase (Within Subjects Effects, "time×plots" effect: df = 156, 312, $F = 5.5$, $p < 0.0001$), when the plots containing *Anthyllis* plants (especially AnFK, AnF and AnK, with An for *Anthyllis*, F for *Festuca* and K for *Koeleria*) showed a very high biomass production (Figure 1). At the final harvest, the plots where *Anthyllis* was initially present had the highest biomass (Figure 2a) and cover rates (Figure 2b), particularly in association

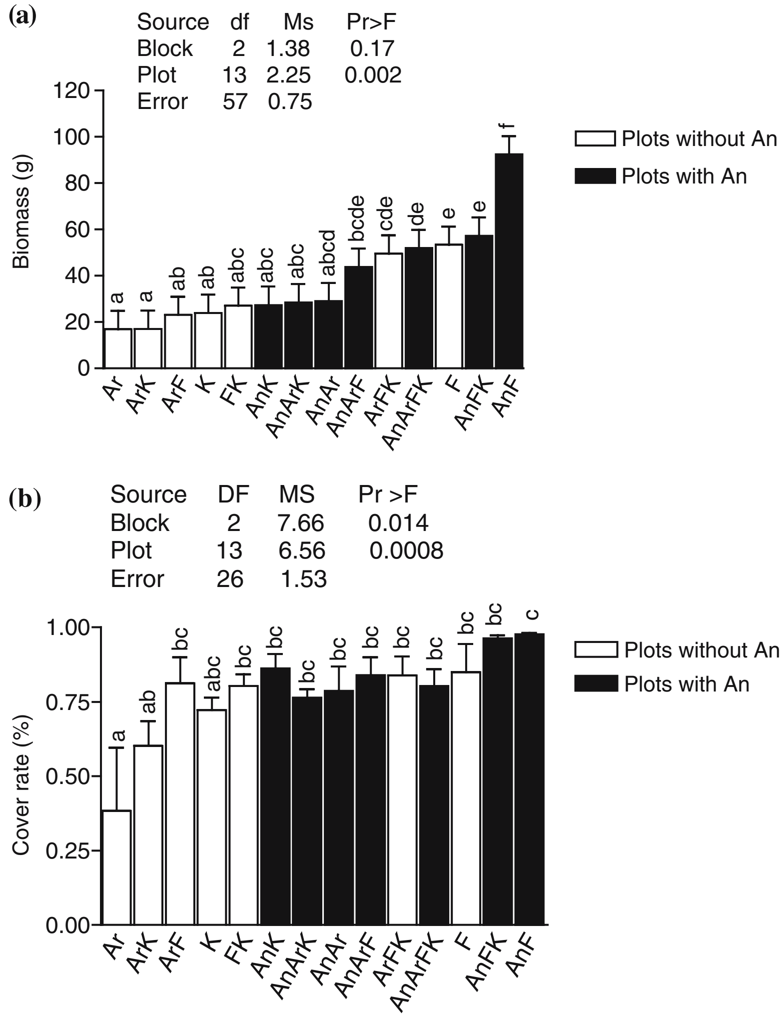

Figure 2. ANOVA and means ± SE of final aerial biomass (a) and the cover rate (b) at the time of harvest, for the 15 plots of different species mixtures. Means with the same letters do not differ significantly at the 5% level with a Tukey test. An: *Anthyllis vulneraria*; Ar: *Armeria arenaria*; F: *Festuca arvernensis*; K: *Koeleria vallesiana*.

with *Festuca* (AnF mixture). In contrast, the plots with *Armeria* monocultures produced the lowest biomass values. Plots with *Koeleria* showed intermediate values.

Flower production

Despite the high soil metal content in the plots, the four species flowered in June 2003 (Figure 1). There was a significant general effect of mixture type on flowering capacity (Figure 3). In particular, flowering of *Anthyllis* individuals increased in the presence of both grasses (F and K) and decreased in plots with *Armeria* (Figure 3a). The presence of the legume did not seem to clearly increase the flowering of the other three species (Figure 3b, c, d).

Anthyllis seedling production

The number of seedlings increased from March to April and decreased in June (Figure 4). There was no significant difference between AnF, AnK and AnFK ($p > 0.38$) at the final harvest. As for

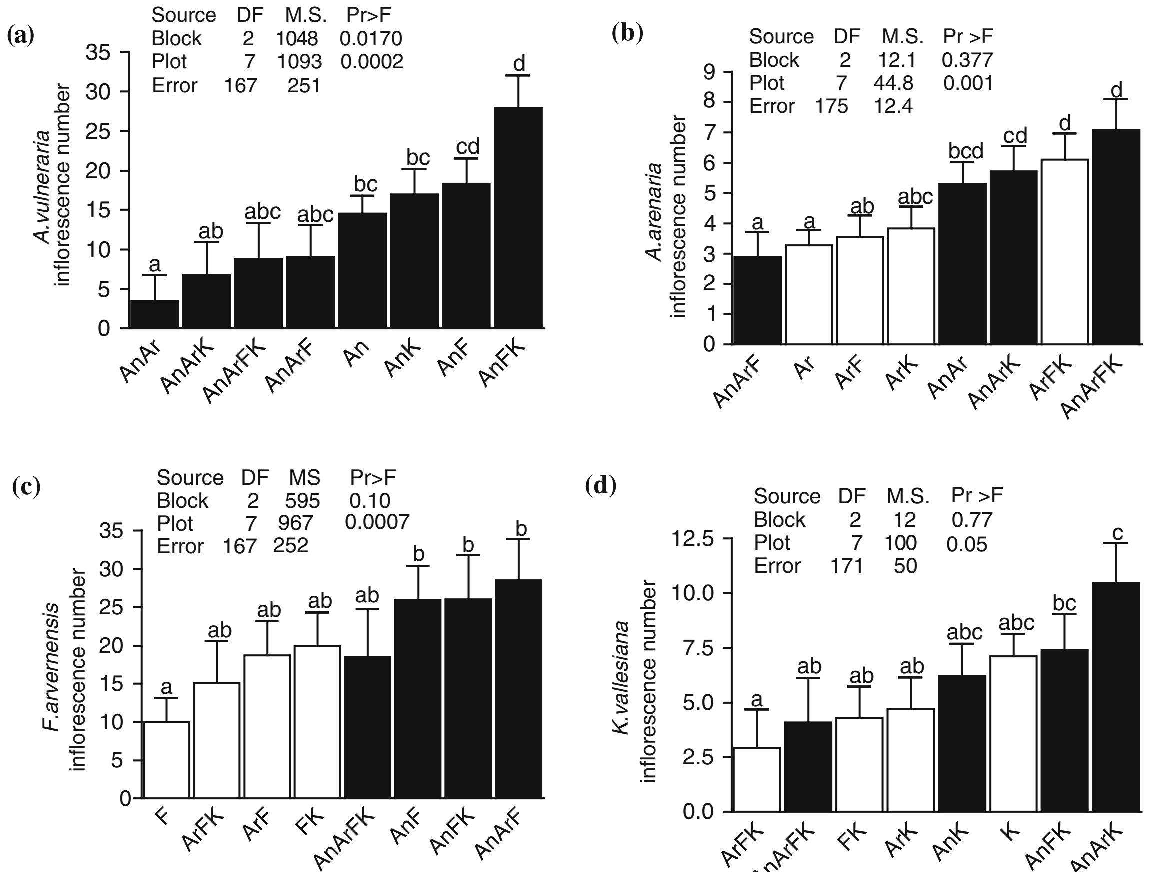

Figure 3. ANOVAs and mean numbers ±SE of inflorescences for (a) *Anthyllis*, (b) *Armeria*, (c) *Festuca* and (d) *Koeleria* individuals in the different plots containing these species, recorded at the first flowering peak (June 2003). For each species means with the same letters do not differ significantly at the 5% level with a Tukey test. An: *Anthyllis vulneraria*; Ar: *Armeria arenaria*; F: *Festuca arvernensis*; K: *Koeleria vallesiana*.

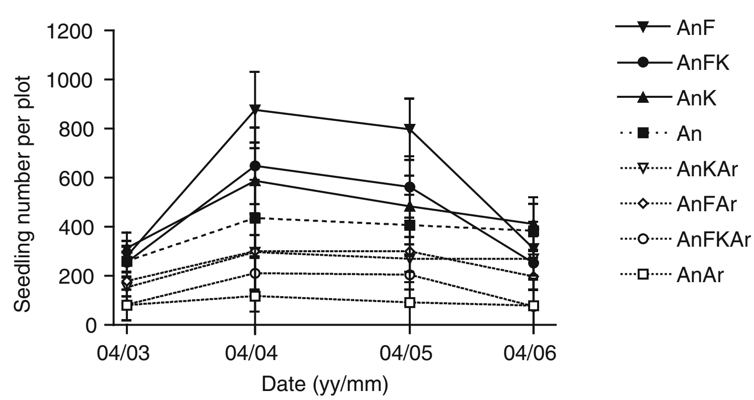

Figure 4. Mean numbers ±SE of *Anthyllis vulneraria* seedlings per plot recorded during the last four months of the experiment in plots where this species was initially present. Continuous lines represent plots with the two grasses, *Festuca arvernensis* and *Koeleria vallesiana*, dotted lines the plots with grasses and *Armeria arenaria* individuals, and the thick dotted lines the plots with initial monocultures of *Anthyllis vulneraria*. An: *Anthyllis vulneraria*; Ar: *Armeria arenaria*; F: *Festuca arvernensis*; K: *Koeleria vallesiana*.

the biomass values, the presence of *Armeria* in the mixtures appeared to have a negative effect on the regeneration of *Anthyllis*.

Metals and nutrients

Horse manure did not change the concentration of available metals in the soil compared to the contaminated soil of the site, probably due to the relatively low level of its input in weight (Table 2A). There were also no differences in soil metal content among plots with monocultures (results not shown). The results also showed that plots with *Anthyllis* were not different from the other plots except for nitrogen, which showed a significant increase (Table 2A). Indeed, the values for the monocultures and binary mixtures indicated that the N and P concentrations were increased in the aerial tissues for the three species in plots where *Anthyllis* was initially present compared with monocultures (Table 2B). There were also significant differences between species for all elemental concentrations (*Anthyllis* plants were not analysed because they disappeared after flowering). *Armeria* generally showed the highest values compared with the two grasses, except for Pb (Table 2B).

However, when only the two grass species were considered, they showed significant differences in some elements including metals when growing with *Anthyllis*. For example, *Festuca* and *Koeleria* individuals had higher concentrations of K ($p < 0.05$) and low concentrations of Zn ($p < 0.05$), Cd ($p < 0.05$), and Pb ($p < 0.05$) in plots where *Anthyllis* was initially present, compared with the monocultures (Table 2B).

Discussion

The need to use metallicolous individuals from local species

The results clearly showed that the populations of *Anthyllis*, *Festuca* and *Armeria* from the heavily polluted site of "Les Avinières" are more tolerant to zinc than the populations from uncontaminated sites. This confirms that zinc tolerance has an adaptive basis in these species. In addition, it is highly probable that metallicolous individuals of *Koeleria*, that were not tested in nutritive solution, would have shown the same zinc tolerance level as metallicolous individuals of *Festuca*, as the survival rates of metallicolous individuals of *Festuca* and *Koeleria* in the field experiment were similar. The usefulness of metal-tolerant plants from mine spoils for the phytoremediation of contaminated sites has been demonstrated since the end of the 1960s in England (Gadgil, 1969; Smith and Bradshaw, 1970). However most of the British sites had contamination levels lower (for example 9000 mg/kg total Zn in soils analysed by Gadgil, 1969) than the site of Avinières (160 000 mg/kg total Zn), and were under a wet temperate climate, contrasting drastically from the Mediterranean environment, where species have to cope with the long dry summer season.

From the field trials, it was showed that in spite of the very high metal toxicity (Zn, Pb, Cd) and the harshness of the dry season, the survival, growth and flowering of the four metallicolous ecotypes of the local species under investigation were insured. Therefore, this work emphasises the need to conserve locally growing metallophytes so as to include them in the restoration efforts for industrial or mine soils after the end of the human activity (Whiting et al., 2004).

Moreover, Gadgil (1969) and Smith and Bradshaw (1970) also showed that the input of fertilizers significantly increased the yield of the plants tested in the mine trials. Previous studies demonstrated that the growth of plants on toxic soils was significantly higher with compost addition (Frérot, 2004). Since these trials were performed on a bare toxic substrate, the data obtained underscore the efficiency of organic fertilisation in the phytostabilization process.

The improvement of phytostabilization due to facilitation effects

Another important result is the positive interactions between the Fabaceae *Anthyllis* and the two grass species. *Anthyllis* increased the growth of *Festuca*, particularly in the binary mixture An + F where the biomass of *Festuca* was the highest among all the treatments. *Festuca* and *Koeleria* in co-culture with *Anthyllis* showed an increased concentration of essential nutrients (N P K) and decreased concentrations of heavy metals (Zn Pb Cd) in their leaves when compared

Table 2. Mean values (Standard Errors) of soil concentrations (A) in plots with or without *Anthyllis*, and of mean elemental concentrations (B) in shoots of the three species (F = *Festuca arvernensis*; K = *Koeleria vallesiana;* Ar = *Armeria arenaria*) in binary mixtures with *Anthyllis vulneraria* (+An) or in monocultures after two years of growth. (A) ANOVA Soil. Differences between plots for N: $F = 6.3$ df = 2,14 $p < 0.01$. Other elements: non significant. (B) ANOVA Plants. An: significance of differences between plots with and without *Anthyllis* in mixtures; Sp: significance of differences between species F, K, Ar with and without *Anthyllis*. There were no significant interactions. Comparison of means: Tukey tests of mean differences between plots for the different elements. $N = 3$ for the plant analysis. For each column, means with the same letters (abcd) do not differ significantly at the 5% level

(A) Soil								
	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Zn (mg kg^{-1})	Pb (mg kg^{-1})	Cd (mg kg^{-1})
Soil $n = 3$	0.042 (0.006) a	7.9 (3.9)	150 (71)	4734 (1150)	303 (37)	29279 (2975)	40387 (1722)	360 (34)
Soil + Horse manure $n = 9$	0.047 (0.002) a	8.5 (1.6)	240 (82)	6218 (2030)	371 (18)	27537 (1501)	51653 (3790)	569 (18)
Soil + Horse manure + *Anthyllis* $n = 9$	0.070 (0.005) b	8.9 (1.5)	158 (25)	2836 (654)	177 (13)	25709 (1547)	48664 (2536)	304 (17)
(B) Plants								
	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Zn (mg kg^{-1})	Pb (mg kg^{-1})	Cd (mg kg^{-1})
ANOVA								
An	$P = 0.001$	$p = 0.007$	$p = 0.15$	$p = 0.40$	$p = 0.18$	$p = 0.36$	$p = 0.38$	$p = 0.95$
Sp	$P = 0.04$	$p = 0.001$	$p = 0.001$	$p = 0.001$	$p = 0.001$	$p = 0.04$	$p = 0.05$	$p = 0.001$
Comparison of means								
F	0.60 (0.06) d	458 (34) c	4080 (149) c	3171 (298) b	822 (121) b	2885 (721) ab	1002 (297) ab	19 (7) bc
F (+An)	0.76 (0.07) cd	749 (144) c	6257 (494) c	2281 (211) b	624 (82) b	1469 (292) b	376 (101) b	8 (2) c
K	0.98 (0.18) bcd	615 (12) c	6819 (404) c	4320 (79) b	1095 (134) b	3514 (270) ab	1960 (61) a	34 (4) abc
K (+An)	1.62 (0.39) abc	1082 (152) bc	10193 (1650) bc	4464 (276) b	1359 (253) b	2786 (499) ab	1477 (365) ab	26 (3) bc
Ar	1.90 (0.12) ab	1461 (83) ab	17029 (1551) a	11223 (1580) a	4818 (406) a	5427 (1150) a	1605 (603) ab	66 (15) ab
Ar (+An)	2.22 (0.24) a	1864 (219) a	16624 (2278) ab	10206 (1442) a	3812 (342) a	5748 (1053) a	1787 (542) ab	84 (17) a

with the monocultures. This could explain the increase in biomass of both grasses in the mixtures with *Anthyllis*. In contrast, the mixtures with *Armeria* produced the lowest biomass values.

It is well known that the use of legumes to revegetate derelict land is one of the most valuable tools for phytoremediation as they are a source of nitrogen for the other members of the plant community (Jefferies et al., 1981). However, as legumes are not frequent spontaneous colonizers of highly heavy metal contaminated areas in Europe (Harris et al., 1996), metallicolous individuals of *Anthyllis vulneraria* are particularly interesting as potential facilitators for other species in the phytostabilization process.

The low zinc tolerance of *Anthyllis* relative to the other three non-legume species tested is in agreement with data from Bradshaw and Chadwick (1980), who showed that white clover (*Trifolium repens*) is more sensitive to metal toxicity than grasses such as ryegrass (*Lolium perenne*). It is possible that high concentrations of Zn and Cd can restrict nodulation of legume species (El-Kenawy et al., 1997), or that *Rhizobium* within the root nodules becomes ineffective in N_2 symbiotic fixation (McGrath et al., 1988). However, ongoing research on *Anthyllis vulneraria* (Cleyet-Marel, personal communication) has shown that *Anthyllis* from "Les Avinières" tailings are nodulating in association with a still non-identified genotype of *Rhizobium* tolerant to high metal concentrations and effective in nitrogen fixation. Healthy individuals from our experimental plots indeed showed abundant nodulation compared with chlorotic or dead individuals (data not shown). This shows that the *Rhizobium* population from "Les Avinières" contains strains tolerant to heavy metals and associated with *A. vulneraria* as it has been found in *T. repens* (Delorme et al., 2003; Smith and Giller, 1992).

As *A. vulneraria* is the most abundant legume species in one of the most polluted sites of the Languedoc-Roussillon Region, a key factor for estimating the actual efficiency of any mixture including this species is its persistence over the years. Our results showed that the regeneration of *Anthyllis* was highest in treatments with grasses probably because the cover provided by the grasses facilitated the germination of *Anthyllis* seeds and the survival of seedlings. Conversely, *Armeria* reduced the recruitment of *Anthyllis* seedlings. This could be explained by the little area covered by the leaves of *Armeria* which did not provide any protection from water stress to germinating seeds of *Anthyllis*.

Therefore, facilitation processes (Callaway, 1995) where a nurse species protects another during the first development stages are particularly important in the Mediterranean region (Rousset and Lepart, 2000; Sans et al. 1998) and can be extended to experimental trials in phytostabilization.

Conclusion

Despite the high metal concentration and the water shortage at the heavy metal polluted Mediterranean mine site of "Les Avinières", it was showed that the use of metallicolous ecotypes from local species combined with the addition of organic manure facilitated the revegetation of bare areas. The use of a legume species such as *Anthyllis vulneraria* in mixture with non-legume species increased the biomass of the other species and consequently the biomass production of the plant community. This results from the effective nitrogen fixation by a tolerant and still undetermined genotype of *Rhizobium* in symbiosis with *A. vulneraria*, which improves soil fertility. Moreover, the regeneration of *A. vulneraria* is effective as seeds germinate and seedlings establish under the cover of grasses by a probable facilitation effect of the latter. On the contrary, *Armeria* appeared to be detrimental for the establishment of the grasses–legume community. Therefore a successful phytostabilization project in the Mediterranean region needs an association between grasses and a legume, where grasses form a dense plant cover facilitating the seedling recruitment of *Anthyllis*, which in turn, at the adult stage, enhance the growth of the grasses. Thus, the mixtures of one or two species of grasses with *Anthyllis* could constitute a suitable species assemblage in which positive interactions on survival, growth, and regeneration promote persisting plant communities.

Note

[1] Type III sums of squares were used in all analyses of variance.

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