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RESEARCH ARTICLE

Local Adaptation of Metallicolous and Non-Metallicolous *Anthyllis vulneraria* Populations: Their Utilization in Soil Restoration

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Abstract

Restoration of metalliferous mine soils requires using plant species tolerant to high metal concentrations and adapted to nutrient-poor soil. Legumes can increase plant productivity through N_2-fixation, but they are often scarce in metalliferous sites. We examined survival, growth, and tolerance of four populations of a legume, *Anthyllis vulneraria*, from two metalliferous (MET) Zn-Pb mine sites, Avinières (AV) ([Zn-EDTA] = 26,000 mg/kg) and Eylie (EY) ([Zn-EDTA] = 4,632 mg/kg), and two non-metalliferous (NMET) sites located in the south of France with the aim to select the most appropriate populations for restoration of mined soils. In a common garden experiment, plants from each population were reciprocally grown in soil from the provenance of each population. The two NMET populations exhibited high mortality and low growth rates in soil from the mined sites. The AV MET exhibited a high growth rate in metalliferous soils, but showed high mortality in non-metalliferous soils. The growth of the EY MET was very low in the AV-contaminated soil, but was the highest of all populations in moderately and non-metalliferous soils. Plants from the AV MET population showed a high growth and survival in metalliferous soil and would be appropriate in the restoration of metal-contaminated sites (>30,000 mg Zn kg^{-1}). The EY MET population would be adapted to the restoration of moderate metal-contaminated soils (<30,000 mg Zn kg^{-1}). Taking into account the broad distribution of *A. vulneraria*, these two populations could be suitable for the restoration of derelict mine sites in mediterranean and temperate regions of Europe and North America.

Key words: **legumes, metal-tolerant species, mine tailings, phytostabilization.**

Introduction

Soils rich in naturally occurring heavy metals—such as ultramaphic soils—and those contaminated with metals due to human activity—such as mining—can be extremely toxic to animals and plants (Shaw 1990). Phytostabilization is an effective and non-intrusive technology that involves the use of metal-tolerant plants to reduce erosion and dispersal of toxic elements from contaminated soil into the environment (Salt et al. 1998). Because major nutrients (N, P, and K) are frequently in short supply in mine soils (Bradshaw & Chadwick 1980), legumes are used to increase the soil fertility and plant productivity through N_2 fixation (Vitousek and Field 1999).

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A general guideline in ecological restoration is to use local species (McKay et al. 2005) because they are assumed to be locally adapted (Leimu & Fischer 2008; Bischoff et al. 2010). However, only a few species within local floras are tolerant enough to colonize metal-contaminated mined sites (Antonovics et al. 1971). Furthermore, legume species are very rare in contaminated soils because metals are often toxic to symbiotic rhizobia (Chaudri et al. 2008). Therefore, using non-local legume species may be needed to phytostabilize and increase the fertility of mine soils.

The symbiotic association between the legume *Anthyllis vulneraria* L. and a new species of rhizobia (*Mesorhizobium metallidurans*) on an abandoned mine at Saint Laurent le Minier (southern France), tolerant of the high soil metal concentrations of Zn, Pb, and Cd (Vidal et al. 2009), has been used for the phytostabilization of mine tailings (Frérot et al. 2006). Mahieu et al. (2011) demonstrated that 80% of total N in *A. vulneraria* originated from atmospheric N_2. This species accumulated Zn in aerial parts (Escarré et al. 2011), which can be toxic to herbivorous animals. Nevertheless, Zn from leaves returning to the soil at plant senescence is negligible in comparison to high soil concentrations. This monocarpic species dies after flowering and releases around 400 kg/ha^{-1}

of N into the soil through rhizodeposition and decomposition of senesced tissue after 2 years growth (Mahieu unpublished data).

There are other *A. vulneraria* populations on non-metalliferous soils in the region around the two mine sites. If metal tolerance is present in some of these populations, they could potentially be used for phytoremediation of metalliferous land. The species is predominantly selfing and easy to multiply because protandrous flowers prevent cross fertilization. Conservation of these metal-tolerant populations is imperative (Whiting et al. 2004), but it is also essential to investigate the ability of *A. vulneraria* populations to survive and grow under conditions other than those of their native environment.

We performed a reciprocal transplant experiment with four geographically distinct populations of *A. vulneraria* (two metallicolous and two non-metallicolous) to evaluate their survival and growth in metalliferous and in non-metalliferous soils, and to test whether non-metallicolous populations have a constitutive tolerance to metal-contamination. Each population was cultivated in its soil and in the soils of each of the other three populations. Since *A. vulneraria* is a Zn accumulator, the metal concentrations in the above and belowground parts of plants were measured. Given the large distances between the populations and for logistical and ethical reasons, we conducted research under controlled conditions following the "explant" approach (Kawecki & Ebert 2004).

Methods

Anthyllis vulneraria L. is a rosette-forming legume of 15–30 cm height with 24 subspecies in Europa (Tutin et al. 1964–1993). Although predominantly selfing, allogamy is possible because of a low proportion of male sterile individuals (Couderc 1971). The species is present in Europe, Asia, North Africa, and naturalized in North America. *A. vulneraria* prefers the calcareous grasslands and rocky environments, but also grows in metalliferous soils.

Two non-metallicolous (NMET) and metallicolous (MET) populations of *A. vulneraria* were used in the experiment. The first non-metallicolous *A. vulneraria* population belongs to the subspecies *vulneraria* and was collected near St. Chély (SC) on the Causse Mejean (03°23′51″E; 44°18′20″N), a limestone plateau in southern France situated at an altitude of 800–1,250 m. The climate was temperate, with a mean annual rainfall of 920 mm (Meteo France, 1986–2005). Soils were sandy on dolomite bedrock (Cadillon 1970). The second non-metallicolous population belongs to subspecies *praepropera* and was collected near Col du Vent (CV) on the Causse du Larzac plateau (03°27′57″E; 43°45′53″N), an outcrop situated at an altitude of 560–920 m on the southern margin of the Massif Central. Soils were derived from dolomitic bedrock (Cadillon 1970) and the climate has both oceanic and Mediterranean influences, with annual precipitation between 710 and 1730 mm (Meteo France, 1988–2005).

The two metallicolous populations of *A. vulneraria* originated from metal-contaminated soils as a result of old mining activities. The first one belongs to subspecies *carpatica* and was collected from the Avinières (AV) mine, which was abandoned at the beginning of the 20th century. The mine site

Table 1. Chemical characteristics and total and pseudo-total EDTA-extracted concentrations (mg/kg) of Zn, Pb, and Cd determined on the fine earth fraction (<2 mm) of the studied soils.

	Metalliferous soils			*Non-metalliferous soils*	
	Avinières 90[a]	*Eylie 100*[b]	*Eylie 90*[a]	*Col du Vent*	*St. Chély*
pH_{water}	8.2	8	8.5	8.4	8.4
Organic matter status (g/kg)					
Organic matter	33.5	8.6	9.1	53.3	53.5
N total	0.6	0.4	0.7	2.7	2.2
C/N	31.0	17.2	22.1	11.5	14.1
Extractable metal (mg/kg)					
Cd-EDTA	430	16	n.d.	1	4
Pb-EDTA	49,000	4,158	n.d.	20	106
Zn-EDTA	26,000	4,632	4,667	59	114
Total metal concentrations (mg/kg)					
Cd	1,210	51	50	1	4
Pb	34,212	10,014	9,566	59	106
Zn	173,263	6,490	6,236	233	585

n.d., not determined.
[a] 10% compost and 90% mine soil.
[b] 100% mine soil.

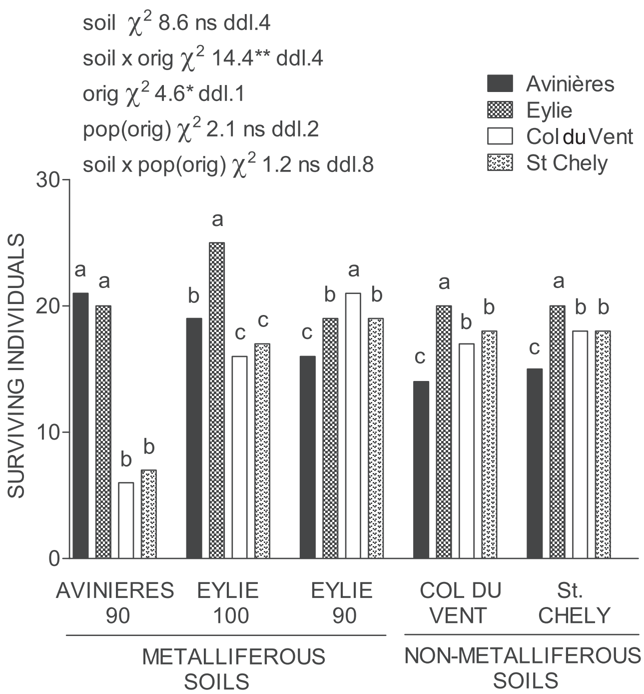

Figure 1. Survival (%) of *Anthyllis vulneraria* individuals from four populations originating in metalliferous soils from Avinières and Eylie, France and non-metalliferous soils from Col du Vent and Saint Chély, France. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences ($\alpha = 0.05$) within each of the five soil treatments were denoted by the use of different lowercase letters (*$p < 0.05$; **$p < 0.01$; ns, not significant; *df*, degrees of freedom).

was located at Saint Laurent le Minier, 40 km north-west of Montpellier (03°39′39″E; 43°55′57″N). The population was comprised of greater than 1,000 individuals, but the species was not present in the other mine sites of the region (J. Escarré 2011, CEFE, UMR CNRS 5175, Montpellier, France, personal observation). Average annual rainfall was of 1,504 mm (Meteo France, 1971–2000), with maximum occurring in spring and autumn and minimum occurring in summer. The mine spoil from AV was dominated by dolomitic rock. Soil was collected from the 0–15 cm depth from the tailing ponds located below the mine spoils along the Vis River valley.

The second metallicolous population belongs to subspecies *boscii*. This population was collected from the clay-rich mine waste heaps abandoned since 50 years at Eylie (EY), 95 km south-west of Toulouse (00°56′14″E; 42°50′08″N). Average annual rainfall was 1,027 mm (Meteo France, 1971–2000), evenly distributed throughout the year. Population size is less than 1,000 individuals (J. Escarré 2011, CEFE, UMR CNRS 5175, Montpellier, France, personal observation).

Soil chemical characteristics are given in Table 1.

Soils from all four sites were collected near patches of *A. vulneraria* transported to the laboratory, dried, and sieved (2-mm mesh). Seeds were germinated on moisturized paper in Petri dishes. At the cotyledon stage, seedlings were transferred to vermiculite before being transplanted individually into 2-L containers filled with the soils from the four provenances (two metalliferous and two non-metalliferous). Because the AV soil was extremely toxic and devoid of nutrients, a mixture of 10% compost and 90% toxic soil (AV90) was used.

Soil toxicity of the EY was unknown so two mixtures were tested: (1) 10% compost and 90% mine soil (EY90) and (2) 100% EY mine soil (EY100). Twenty-five plants

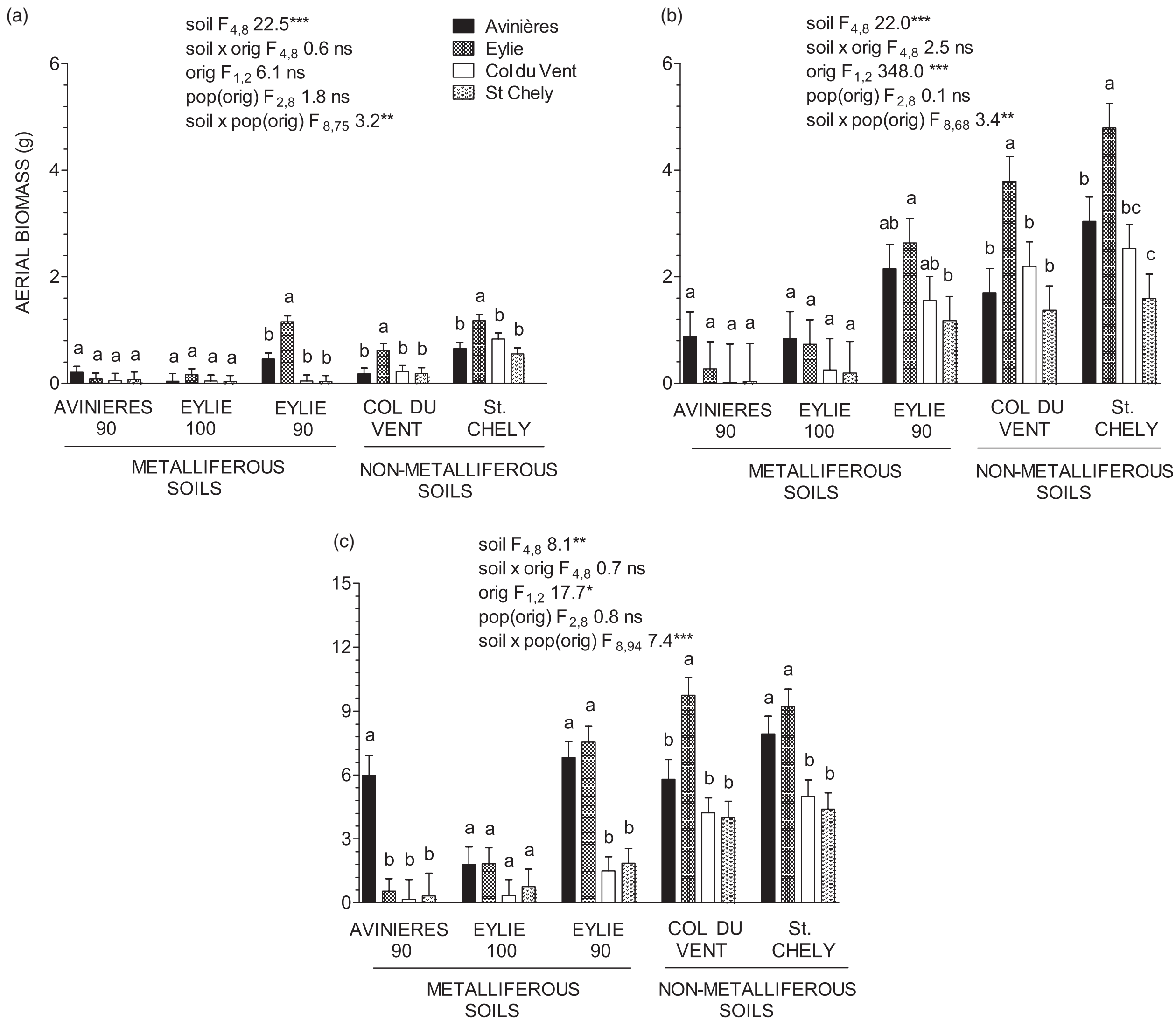

Figure 2. Aerial biomass (mean ± SE) of four populations of *Anthyllis vulneraria* grown in five different soils collected at (a) 130, (b) 170, and (c) 268 days. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences ($\alpha = 0.05$) within each of the five treatments were denoted by the use of different lowercase letters (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

per population and soil origin combination (five soils × four populations) were tested. Pots were randomized in a non-heated greenhouse and watered with deionized water. Plants that died during the first 2 weeks were replaced.

Three to five plants per treatment (depending of the NMET mortality in toxic soils) and population were harvested after 130 (young plants), 170 (adult rosettes), and 268 days, at which point the experiment was stopped due to the death of non-metallicolous plants in toxic soils. Plant material was separated into aerial parts and roots. Aerial biomass was rinsed in deionized water and roots were washed with a solution of $CaCl_2$ 0.5 mmol/L^{-1} at 4°C for 20 min (Papoyan et al. 2007). After being dried at 60°C for 3 days, plant matter was weighed and Zn concentrations of aerial and subterranean parts were determined by the zincon method (Macnair & Smirnoff 1999) based on UV-visible spectrophotometry (HELIOS spectrophotometer, Thermo Spectronic, Cambridge, England) using zincon as colored Zn-chelating agent. Dead plants were collected and analyzed in the same manner as living plants.

Statistical Analyses

Plant biomass and Zn content were analyzed using nested analysis of variance (ANOVA), with a logarithmic transformation of data to respect the normality assumption if necessary using the software program SAS (2004). Because of complex interactions among dates, soils, populations, and provenances appeared in the analyses (data not shown), we analyzed the data by each of the three dates using nested

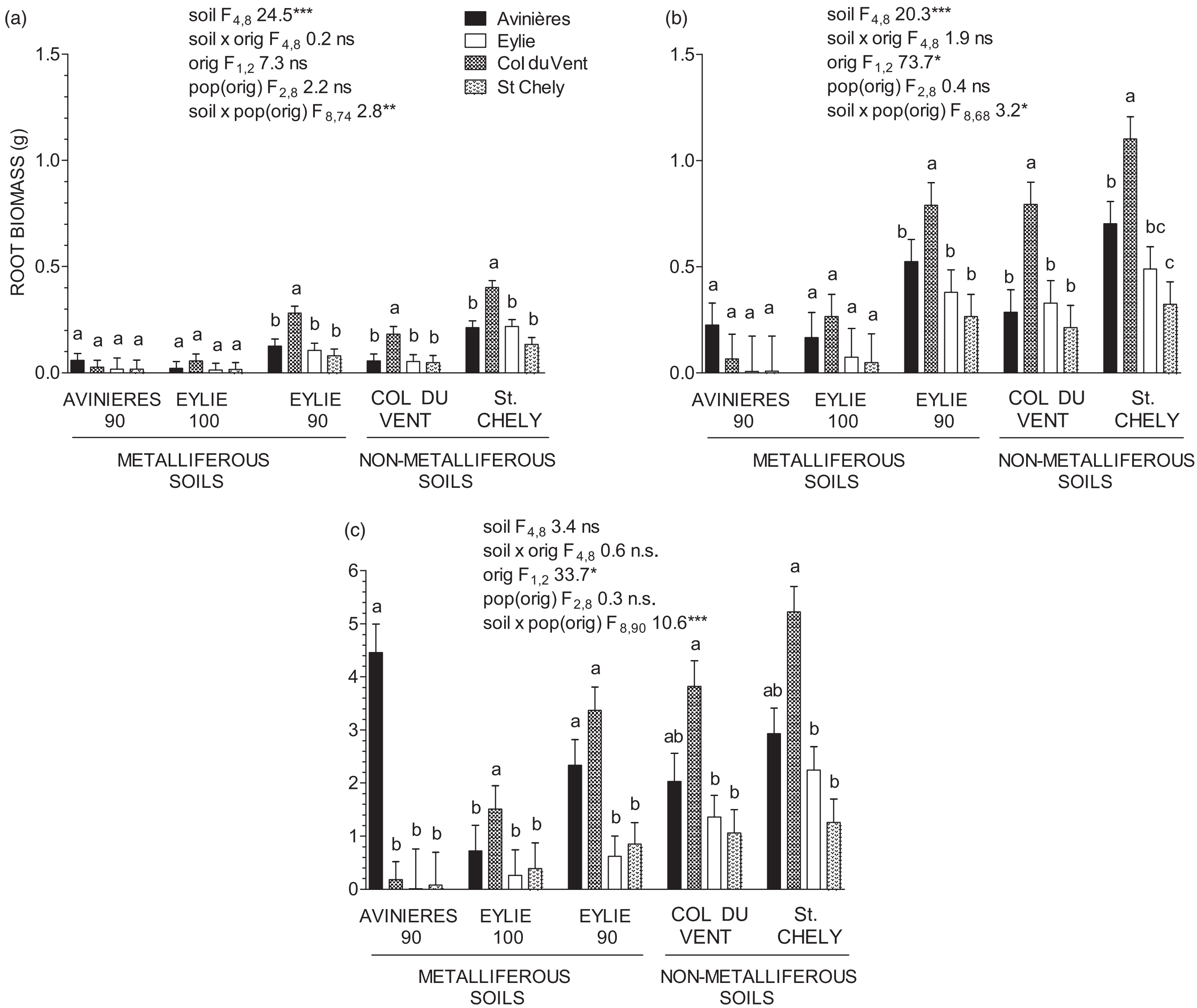

Figure 3. Root biomass (mean ± SE) of four populations of *Anthyllis vulneraria* grown in five different soils collected at (a) 130, (b) 170, and (c) 268 days. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences at the 5% level within each of the five treatments were denoted by the use of different lowercase letters (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. ns, not significant).

ANOVA with the following factors such as soil (five levels), population provenance (MET or NMET), and populations (four levels) nested within the provenance. All factors were considered fixed with the exception of population, which was random. Type III sums of squares were used to estimate F-statistics and degrees of freedom were calculated according to Satterthwaite's approximation. Means were compared with least-squares means tests in SAS (2004). Survival data were analyzed using the GENMOD (SAS 2004) procedure followed by a proportion test for each soil.

Results

Survival rates were significantly different among provenances, near significant ($\chi^2 = 8.6$, $df = 4$, $p < 0.10$) for soil and highly significant for soil × provenance (Fig. 1). Therefore, we analyzed survival differences for each soil. Survival of NMET populations was lower (24–28%) in AV90 relative to the MET populations (80–84%). The EY population had no mortality in its native soil (EY100) and its survival rate was significantly higher than the other populations. Mortality of NMET populations in EY100 soil was less (64–68%) than the mortality on AV90 soil. There were no significant mortality differences among the four populations in both non-metalliferous and EY90 soils. Finally, fewer AV individuals survived in the two non-metalliferous (CV and SC) than in the two most metal-contaminated soils (AV90 & EY100) [58% vs 80%; Fisher Exact test $p = 0.03$].

Non-metalliferous populations displayed low growth in metalliferous soils, although plant biomass increased between

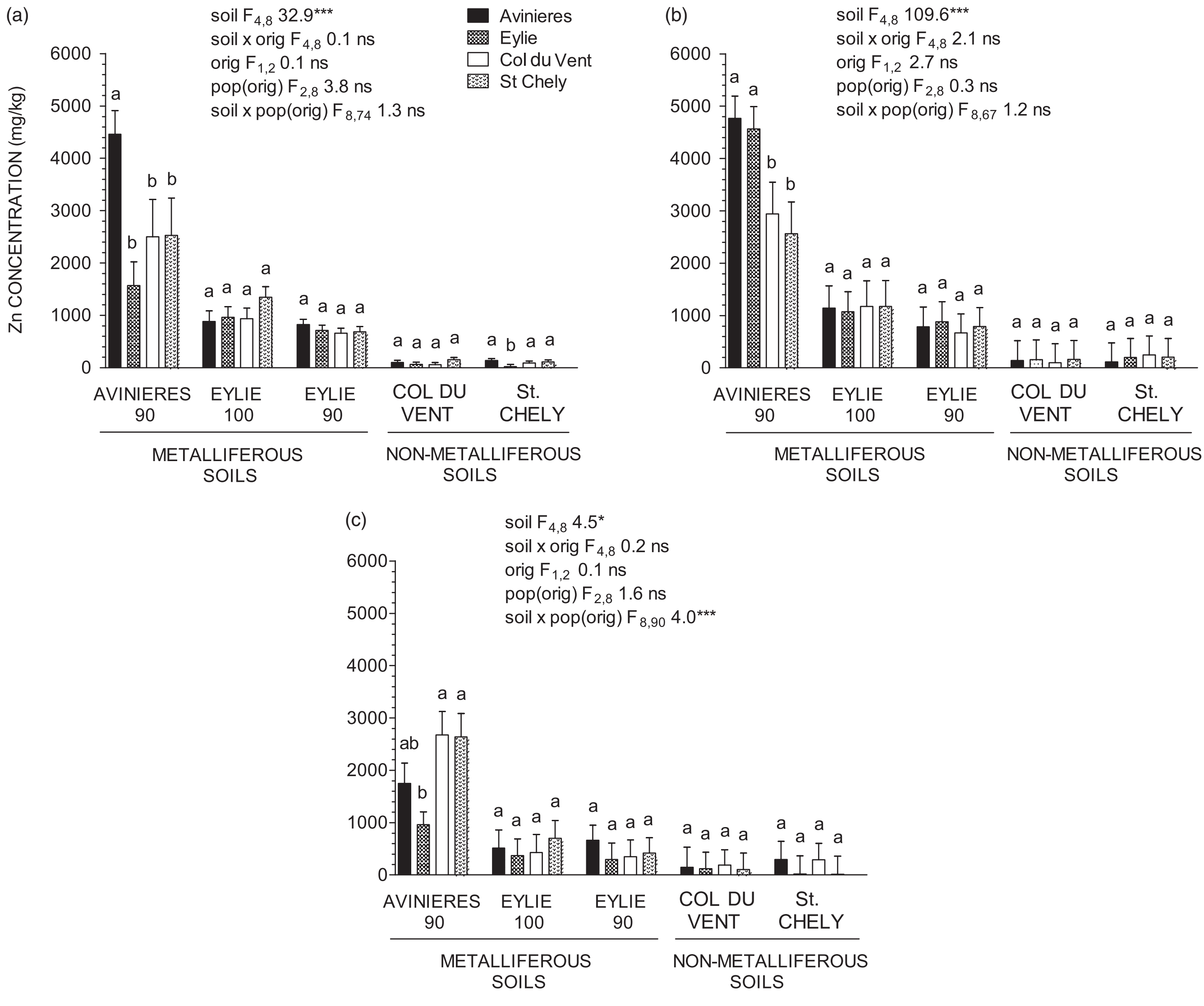

Figure 4. Zinc concentration (mean ± SE) in the shoots of four populations of *Anthyllis vulneraria* grown in five different soils collected at (a) 130, (b) 170, and (c) 268 days. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences at the 5% level within each of the five treatments were denoted by the use of different lowercase letters (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. ns, not significant).

the first and second harvest (Fig. 2a & 2b), but not between the second and third harvest (Fig. 2b & 2c). AV individuals exhibited significantly higher growth at the time of the third harvest compared with that of EY individuals when grown in the former's original (i.e. AV90) soil (Fig. 2c). This difference did not occur in EY100 and EY90 soils where biomass values of the two populations were similar and significantly higher than those of the NMET populations particularly in the third harvest (Fig. 2c). In the two non-metalliferous soils, the EY population had the highest and the NMET populations the lowest biomass values.

The AV population had the highest root biomass in its home soil (Fig. 3b & 3c), whereas the root biomass of the EY population was the greatest in the three less toxic soils (EY90, CV, and SC) and in the EY100 soil at the third harvest (Fig. 3c). The CV and SC populations exhibited the lowest values in their respective soils of provenance (Fig. 3).

Zn aerial concentrations were highest in plants grown in AV90 soil, varying from 1,000 to 5,000 mg Zn kg^{-1} according to the plant subspecies, and were relatively low in the others soils ($\leq$1,000 mg Zn kg^{-1}), including the two from EY (Fig. 4a & 4b). The two MET populations exhibited the highest aerial tissue Zn concentrations at the second harvest, but at the third harvest Zn concentrations had dropped below 2,000 mg kg^{-1}. In contrast, aerial tissue Zn concentrations of NMET populations remained stable at around 2,500 mg kg^{-1} (Fig. 4).

Aerial Zn concentrations in dead plants grown in AV90 soil were extremely high, except for AV (Fig. 5); the latter were characterized by Zn concentrations similar to those of living individuals grown in the same soil (Fig. 4). Dead plants of the three other populations showed high Zn values, with a

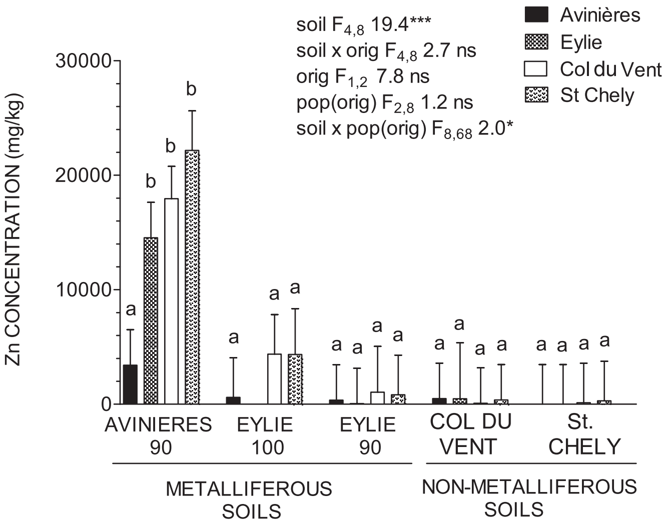

Figure 5. Zinc concentration (mean ± SE) in the shoots of dead individuals of four populations of *Anthyllis vulneraria* grown in five different soils. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences at the 5% level within each of the five treatments were denoted by the use of different lowercase letters (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. ns, not significant).

maximum of 25,000 mg/kg observed in SC individuals. Aerial tissue Zn concentrations of individuals grown in the other soils were similar, although NMET individuals tended to show higher values in the EY100 soil (Fig. 5).

Root Zn concentrations increased over time in plants grown in metalliferous soils (Fig. 6) but remained low in those grown in non-metalliferous soils. There were no differences in Zn concentration among populations for the two first harvests but concentrations in AV plants and those from SC grown in AV90 soil were significantly higher at the third harvest (Fig. 6).

Discussion

High soil metal contamination can exert strong selection in plant populations, which can maintain a local adaptation despite substantial gene flow (Antonovics & Bradshaw 1970). This selection is expected to enhance plant performances in response to local environmental conditions, but may generate trade-offs between tolerance to metals and survival or growth of metallicolous populations in non-metalliferous soils (Antonovics et al. 1971). We found strong differentiation in metal tolerance among subspecies from different provenances, which was related to metal concentration in their native soils. The non-metallicolous populations exhibited a high mortality and low growth rates when grown in soil from the AV mine, with the observed mortality rate due to an excessive accumulation of Zn in plant aerial tissues. The soil from EY and the two (AV90 and EY90) soils improved with 10% compost, remained less fertile than the non-metalliferous soils. Non-metallicolous individuals exhibited lower biomass than those originating from mine soils when grown in non-metalliferous soils. This absence of reciprocal local adaptation was unexpected for a short-lived and mostly self-pollinating species, which are generally thought to be more differentiated compared to those of long-lived species (Linhart & Grant 1996). However, Leimu and Fischer (2008) reported that in experiments investigating reciprocal local adaptation, results in which local populations outperform alien ones in their environment of provenance are less frequent (45.3%) than those in which the alien grows more effectively in both environments (51.4%).

Our results did not validate the hypothesis that metabolic trade-offs would occur between growth in favorable conditions and tolerance to environmental constraints (here metals) (Liancourt & Tielborger 2009). No growth trade-offs were found in either the non-metallicolous EY or (to a lesser extent) AV populations that exhibited similar or higher growth in non-metalliferous than metalliferous soils, suggesting no apparent metabolic cost of plant tolerance at the expense of plant growth. This phenomenon has been explained in *Arabidopsis halleri* by Sarret et al. (2002), who showed that the concentration of organic acids involved in the storage of Zn in the leaf, increased with the concentration of metals in the soil, suggesting that the carbon invested in metal tolerance is available for the plant growth in non-metalliferous soil. However, we showed survival trade-offs in the AV population, which exhibited high mortality in non-metalliferous and in mine soil from

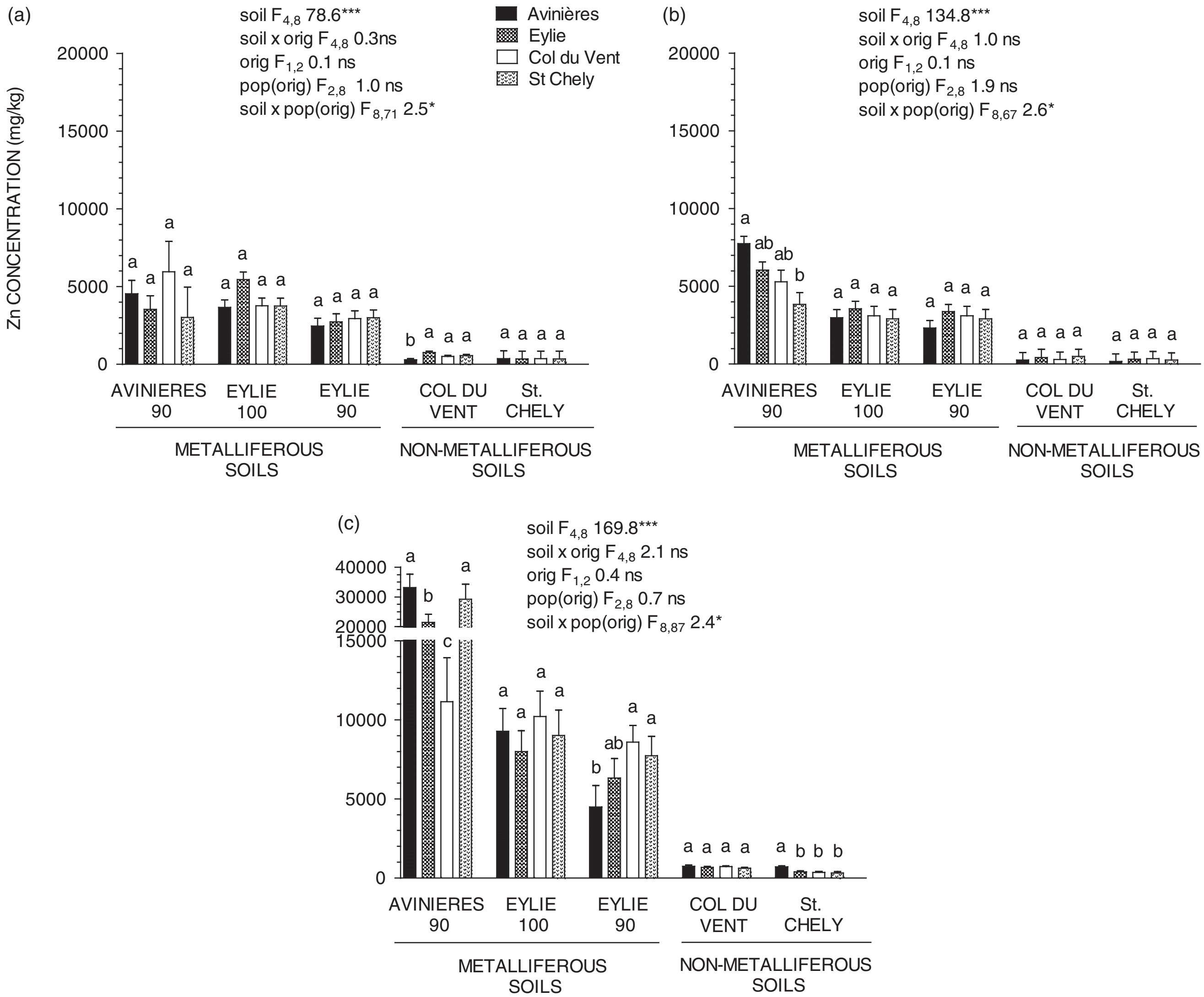

Figure 6. Zinc concentration (mean ± SE) in the roots of four populations of *Anthyllis vulneraria* grown in five different soils collected at (a) 130, (b) 170, and (c) 268 days. "Avinières 90" and "Eylie 90" were a mixed 10% compost and 90% mine soil; "Eylie 100" was 100% mine soil. Significant differences at the 5% level within each of the five treatments were denoted by the use of different lowercase letters (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. ns, not significant).

EY amended with 10% compost; dead plants had low shoot Zn concentrations, suggesting a potential benefit of metal uptake. Most of these plants showed symptoms of pathogen attack that was not present in plants growing in AV soils, which suggests that metals are involved in plant antifungal response, as observed in *A. halleri* and *Thlaspi caerulescens* (Talke et al. 2006; Van de Mortel et al. 2008). Analysis of binary feeding choices in *Anthyllis vulneraria* has shown metallicolous individuals in non-contaminated soils were consumed by gastropods more often than those of non-metalliferous populations ($t = 2.5$ $p = 0.02$, $n = 20$; J. Escarré unpublished data). If local adaptation to soil heavy metal content is a property of metallicolous populations such as that of AV, plants in non-metalliferous environments could be selectively infected and consumed by pathogens and herbivores, respectively.

In metal-contaminated soils, a restricted number of species called hyperaccumulators are able to accumulate large quantities (>10,000 mg/kg) of metals such as Zn in their aerial parts (Baker & Walker 1990). In this study, we showed that metallicolous *A. vulneraria* was not a Zn hyperaccumulator species because the measured Zn concentrations in aerial tissues were below the hyperaccumulation threshold. We observed high variability in Zn concentration among individuals of the metallicolous EY population and the non-metallicolous populations when grown in the AV soil amended with 10% compost: (1) dead individuals with shoot Zn concentrations above 10,000 mg/kg and (2) living individuals with shoot Zn concentrations below 5,000 mg/kg. Mortality in the metallicolous EY and the non-metallicolous populations occurred suddenly, with leaves changing from green to yellow in few days followed by senescence and dead, probably due to an excessive accumulation of Zn in their aerial tissues. Molitor et al. (2005) also found a 2-fold variation range in Zn accumulation in a single population of *T. caerulescens*, which is suggestive of substantial genetic variation in terms of plant Zn accumulation. This suggests that tolerance and hyperaccumulation are negatively correlated both in *T. caerulescens* and in the metallicolous *A. vulneraria* population from EY and in the two non-metallicolous populations.

To our knowledge, this is one of the first studies to demonstrate a genetically based adaptation of a legume to an extreme heavy metal-contaminated soil and provides strong evidence for an adaptive response of the two metallicolous populations to their local soil. In addition, an essential feature of these two populations is their uniqueness because legumes are very rare in environments with metalliferous soils. These characteristics should therefore be taken into account for their conservation. These results have important implications for the restoration of contaminated sites because soils are deficient in nutrients and plants from *Fabaceae* family can increase the nitrogen content of soil and facilitate the colonization by other plant species (Frérot et al. 2006; Mahieu et al. 2011). Taking into account the broad global distribution of *A. vulneraria*, the two metallicolous subspecies could be used for the restoration of mine sites in mediterranean and temperate regions of Europe and North America.

Implications for Practice

- Individuals of the *Anthyllis vulneraria* subspecies *carpatica*, from the Avinières mine, have high tolerance to metals, and would be suitable for restoration of sites with high metal contamination ($>$30,000 mg Zn kg^{-1}).
- The subspecies *boscii*, from Eylie would be better adapted to restoration of moderately contaminated soils ($<$30,000 mg Zn kg^{-1}), which is probably the most common condition prior to restoration.
- The subspecies *vulneraria* and *praeopera*, from non-metalliferous soils were not tolerant to metals and not suitable for the restoration of mine soils.

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