The leguminous species Anthyllis vulneraria as a Zn-hyperaccumulator and eco-Zn catalyst resources

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Abstract *Anthyllis vulneraria* was highlighted here as a Zn-hyperaccumulator for the development of a pilot phytoextraction process in the mine site of Les Avinières in the district of Saint-Laurent-Le-Minier. *A. vulneraria* appeared to hyperaccumulate the highest concentration of Zn in shoots with a better metal selectivity relative to Cd and Pb than the reference Zn-hyperaccumulator *Noccea caerulescens*. A bigger biomass production associated to a higher Zn concentration conducted *A. vulneraria* to the highest total zinc gain per hectare per year. As a legume, *A. vulneraria* was infected by rhizobia symbionts. Inoculation of *A. vulneraria* seeds showed a positive impact on Zn hyperaccumulation. A large-scale culture process of symbiotic rhizobia of *A. vulneraria* was investigated and optimized to allow large-scale inoculation process. Contaminated shoots of *A. vulneraria* were not considered as wastes and were recovered as Eco-Zn catalyst in particular, examples of organic synthesis, electrophilic aromatic substitution. Eco-Zn catalyst was much more efficient than conventional catalysts and allowed greener chemical processes.

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Introduction

Over the past decades, the annual worldwide release of heavy metals in nature has kept increasing (Singh et al. 2003) mostly because of metalliferous mining and smelting from metallurgical industries (Alloway 1995). An illustration of this dramatic situation has been reported in the district of Saint-Laurent-le-Minier, at the mining site of Les Avinières, South of France (03°66′50″E; 43°93′13″N). Mining activities have led to dangerous level of contamination of soil, water, and air causing serious damages to human health, wildlife, flora, and microbe ecosystems. Restoration of polluted sites, as the mining site of Les Avinières, is therefore a current and burning challenge. Different methods are employed to remediate contaminated soils—vitrification, land filling, excavation, and chemical treatments or electrokinetics (Raskin and Ensley 2000). Such chemical and physical methods are definitely costly and irreversibly affect soil properties and biodiversity. Biological and environmental-friendly techniques have thus greatly emerged while being the most cost-effective remediation methods (Padmavathiamma and Li 2007). Phytoremediation that refers to an environment-cleaning technology using plants meets these conditions. Phytostabilization, phytoextraction, and phytofiltration are main techniques of phytoremediation process (Losfeld et al. 2013).

Phytostabilization has been used in the mining site of Les Avinières to stabilize heavy metals in soil with *Anthyllis vulneraria* and *Festuca arvernensis* (Frérot et al. 2006). However, phytostabilization is defined as an adsorption/absorption and accumulation of metals in roots by vacuole sequestration or cell wall binding (Singh et al. 2003; Padmavathiamma and

Li 2007). Metals are not loaded through xylem and are not translocated into the shoots. Using *A. vulneraria* for phytostablizing metals in a contaminated site is thus inappropriate since Escarré et al (2010) have found a high Zn concentration in its shoots. *A. vulneraria* uptakes heavy metals from soil and translocates them in the above-ground parts of the plant, hyperaccumulating them into shoots. Because of the heavy-metal-hyperaccumulating properties, using *A. vulneraria* in phytostabilization could be a concern with metal transfer in the food web and with biomagnification leading to other health risks.

Therefore, we propose here for the first time the utilization of *A. vulneraria* in phytoextraction.

In a phytoextraction process, plants need to be harvested before senescence. However, cut shoots are too frequently considered as wastes. Li et al. (2003) decided to valorise the contaminated biomass in phytomining: metal hyperaccumulating plants were considered as "bio-ores" and treated by conventional mining methods. Phytomining had limited applications, and the economic viability of the process was not evident (Li et al. 2003). Indeed, huge surfaces had been cultivated and led to a low profitability considering the percentage of metals per dry mass in hyperaccumulating plants. Facing this issue, our group has shown that heavy-metal-enriched shoots derived from phytextraction constitutes the starting point of an original and cost-effective approach to modern heterogeneous catalysis, called ecocatalysis (Grison and Escarre 2011; Grison and Escande 2012; Losfeld et al. 2012a, b, c; Escande et al. 2013a, b; Grison and Escande 2013; Grison et al. 2013; Escande et al. 2014; Grison and Escande 2014). Metal hyperaccumulator extracts are transformed in polymetallic ecocatalysts, which are multicomponent chemical systems (Escande et al. 2013a; Grison et al. 2013). Specific interactions and cooperative effects can modulate the overall chemical behavior of the catalysts leading to a high and original catalytic performance. The main goal of this work was to investigate the potential of *A. vulneraria* for large-scale phytoextraction, and its utilization as a green Eco-Zn through pertinent examples.

Material and methods

Material

Reagents and solvents were purchased from Aldrich. Reagents were used without further purification unless otherwise noted. All reactions were performed under argon (or nitrogen) and stirring unless otherwise noted. When needed, glassware was dried overnight in an oven ($T > 100$ °C). All gas chromatography and mass spectrometry analyses were performed using electronic impact (EI) ionization mode on a Varian Saturn 2000 ion trap instrument, interfaced with a Varian CP-3800 apparatus. The Varian CP-3800 was equipped with a 1079 split-splitless injector (206.8 °C) and a 30 m × 0.25 mm × 0.25 mm film thickness ID WCOT CPSil-8CB-fused silica capillary column (Chrompack†, Bergen op Zoom, The Netherlands), with helium as carrier gas (1 mL/min), and programmed 2-min isothermal at 50.8 °C, then increasing from 50 to 220.8 °C at 4.8 °C/min. Mass spectra were recorded in EI at 70 eV and identified by comparison with data of the NIST 98 software library (Varian, Palo Alto, CA) and by comparison of the retention time of the standard compounds. Zn, Cd, and Pb concentrations were measured by atomic absorption spectrometry (AAS) using a spectrometer Thermo iCE 3000 C103500099 v1,30, by pulse polarography and by inductively coupled plasma-mass spectrometry (ICP-MS) Thermo Scientific Elements XR.

Methods

Germination

Seeds of *A. vulneraria* subsp. *carpatica*, collected from Les Avinières mine, near Saint-Laurent-le-Minier were lightly scarified. Different conditions of germination were carried out to get the highest survival rate. When seeds were directly planted in paper, loam/perlite, only 10 % of seeds were germinated after 39 days. When seeds were first transferred in Fleximix Root Riot Organic Starter Cubes©, 42.3 % (SD = 3.03) of seeds were germinated after 15 days. This mixture of coco coir, peat, and bark which has good aeration and drainage properties was then always used for further germination of *A. vulneraria*. Cubes were placed under neon light (about 200 µmol/m^2/s) during 15 days, after what seedlings were transplanted.

Plants cultures

After 15 days, seedlings of *A. vulneraria* were transplanted on "topsoil" culture beds, set up in Les Avinières tailing-ponds. Each topsoil culture bed was made up of two layers. The superior one, of 10-cm height, was composed by soil from the covered part with vegetation and trees of the site. This soil, even though contaminated, allowed a good seedling development, with a low mortality rate after transplantation. The second layer of the topsoil, 5-cm height, was composed by a mix of loam and very contaminated soil (150,000 ppm) of tailing ponds (50/50).

Bacterial culture

Rhizobium metallidrurans ChimEc512[T] was isolated from *A. vulneraria* nodules (Grison et al. 2014b). Fresh culture of *R. metallidurans* was performed in solid medium, yeast extract mannitol (YEM) medium consisting of yeast extract (1 g/

L), mannitol (10 g/L), K_2PO_4 (0.5 g/L), $MgSO_4$ (0.2 g/L), NaCl (0.1 g/L), $CaCO_3$ (1.0 g/L), and agar (15 g/L). After innoculation, Petri dishes were incubated at 30 °C for 48 h to obtain well-grown single colonies. Fourty single colonies were transferred in 16×100 mm test tubes with 4 mL of liquid medium, yeast mannitol broth medium (YMB) consisting of yeast extract (1 g/L), mannitol (10 g/L), K_2PO_4 (0.5 g/L), $MgSO_4$ (0.2 g/L), NaCl (0.1 g/L), and $CaCO_3$ (1.0 g/L). A range of heavy-metal-enriched YMB was tested as follows: 0 mM $ZnSO_4$, 15 mM $ZnSO_4$, 25 mM $ZnSO_4$, 35 mM $ZnSO_4$, and 40 mM $ZnSO_4$. The liquid media was kept at 30 °C and thoroughly agitated at 200 rpm for 5 days.

*Heavy metals analysis of **A. vulneraria** shoots*

A. vulneraria leaves were collected from 500 plants over 5 years of growing on the "Les Avinières" mine site, Saint-Laurent-le-Minier, Gard, France. Leaves were dehydrated for AAS and calcined by pyrolysis for polarography analysis. Pulse polarography was performed according to Golimowski and Rubel in 1974.

*Preparation of Eco-Zn catalyst derived from **A. vulneraria***

Leaves were harvested before flowering, air-dried, and crushed. The obtained solid (150 g) was calcinated at 400 °C for 5 h and the resulting powder (14.8 g) was added to 1 L of a solution of hydrochloric acid (~1 M). The solution was heated at 60 °C and stirred for 2 h. The reaction mixture was then filtered on celite. The resulting solutions, composed of different metal chlorides, were then concentrated under vacuum, and dry residues were stored under nitrogen atmosphere.

*Analysis of Eco-Zn catalyst derived from **A. vulneraria***

ICP-MS analyses were performed using the metal analysis of total dissolved solutes in water. The dry samples were acidified with nitric acid 2.5 %, stirred for 30 min, and diluted to 0.05 g/L. Three blanks are recorded for each step (acidification and dilution) on a HR-ICP-MS Thermo Scientific Element XR.

Bromination experimental procedure

In a typical procedure, the liquid aromatic substrate (5 equiv., 1.96 mmol) was introduced into a 25-mL flask equipped with a magnetic stirring bar. The Eco-Zn supported on montmorillonite K10 (16.44 mg (0.039 mol Zn) of finely ground catalyst in the presence of 80 mg of K10, then dried by heating on electric bill for 15 min at 150 °C), is then added to the starting material while stirring. To avoid any radical bromination, the reaction was carried in the dark. Bromine (1 equiv.,

0.39 mmol) was then introduced at once, while stirring. The reaction was monitored by GC MS analyses and the obtained products were compared to standard references. The reaction was complete in a few hours at room temperature for the compounds that were activated by electron-donating substituents. Good yields were obtained with the deactivated compounds, though heating to 60 °C was provided, water cooler. All the conditions and yields were detailed for each compound in Table 4.

In a typical procedure, the solid aromatic substrate (1 equiv., 1 mmol) was introduced in a 25-mL flask equipped with a magnetic stirring bar. The solid was dissolved in 3 mL of dichloromethane. The Eco-Zn supported on montmorillonite K10 (50 mg of finely ground catalyst in the presence of 80 mg of K10, and then dried by heating on electric lamp for 15 min at 150 °C) is then introduced to the mixture while stirring. To avoid any radical bromination, the reaction was carried in the dark. Bromine (1 equiv., 1 mmol) was then introduced at once, while stirring. The reaction was monitored by GC MS analyses and the obtained products were compared to standard references. The reaction was complete in a few hours at room temperature for the compounds that were activated by electron-donating substituents. Good yields were obtained with the deactivated compounds, though heating to 60 °C was provided, water cooler. All the conditions and yields were detailed for each compound in Table 4.

Results and discussion

The phytoextraction technology is based on Ni-hyperaccumulating plants from the ultramafic soil where nickel is the targeted metal. Experience with Zn-hyperaccumulating

Fig. 1 Inflorescence of *Anthyllis vulneraria* subsp. *carpatica*

Fig. 2 The whole aerial parts of an individual of *Anthyllis vulneraria* subsp. *carpatica*

plants has not been much studied. These species are quite rare and the growing environment is often delicate because Zn-enriched soils are mostly due to mining exploitation leading to the degradation of the natural habitat. The mining site of Les Avinieres, South of France (03°66′50″E; 43°93′13″N), illustrates this problem: the pollution in soil is very high (Zn up to 156,000 ppm, Pb 36,354 ppm, Cd 700 ppm, Tl 115.1 ppm) but phytoavailable (Grison et al. 2010; Escarré et al. 2010). The soil fertility is low, heavy soil texture is not favorable to cultures, and water holding capacity is poor. A known Zn-hyperaccumultor present in this site is *Noccaea caerulescens*. It is a small plant that presents a poor biomass. Another Zn-hyperaccumulating, *A. vulneraria*, has been identified by Frérot et al. (2006) on the pilot site without the drawbacks presented by *N. caerulescens*.

A. vulneraria is undoubtedly an opportunity for Zn phytoextraction, due to the high accumulation ability; the

agronomic abilities, an abundant biomass; and an interesting root system increasing the organic matter and soil fertility as every legume. Indeed *A. vulneraria* harbors a unique rhizosphere that may improve restoration and reclamation activities (Grison et al. 2014b). All the aspects have been first studied.

A. vulneraria, a metal hyperaccumulating legume

About 30 subspecies of *A. vulneraria* have been identified. Most of them tend to grow in the calcareous grasslands and rocky environments, while others are able to grow in metalliferous mine soils. Soil properties remain the same; it is derived from dolomitic limestone for both ecoytpes. A novel distribution of non-metallicolous and metallicolous populations recently identified in the region of Cévennes, South of France, is proposed here. New non-metallicolous *A. vulneraria* populations belonging to subspecies *praepropera* have been observed around the Causse de Blandas: at the south-east extremity at Pic d'Anjeau (867 m, 3°63′06″E, 43°91′69″N); near Saint-Bresson at the "Col des Aires" (520 m, 3°38′36″E, 43°57′14″N); near Beauquiniès (3°61′66″E, 43°89′24″N); around the Cirque de Navacelles (3°51′16″ E, 43°89′42″N); near Saint Jean de Buèges, along Buèges valley (3°62′17″E, 43°82′77″ N); and between the Causse de Blandas and the Causse du Larzac, at the Vis resurgence (3°48′09″E, 43°90′ 53″N). The only metallicolous population found in the area belongs to subspecies *carpatica* (Figs. 1 and 2) and grows in the metal-contaminated soil of the Les Avinières mine site (03°66′50″E; 43°93′13″N). The population was comprised of greater than 1000 individuals, but the species was not present in the other mine sites of the region.

Fig. 3 Comparison of Zn, Cd, and Pb concentrations (ppm) in shoots of *A. vulneraria* and *N. caerulescens*
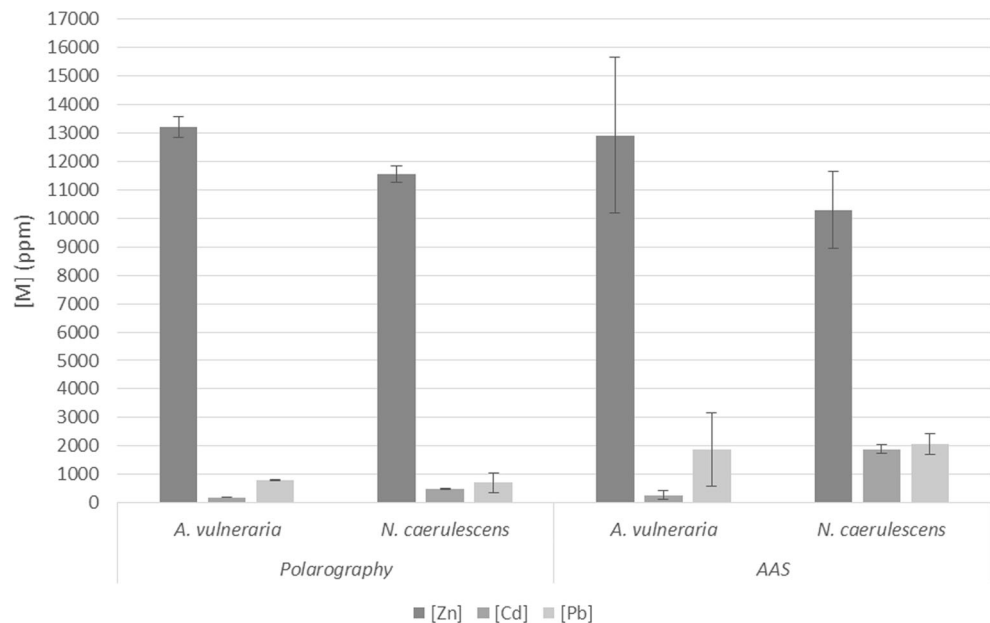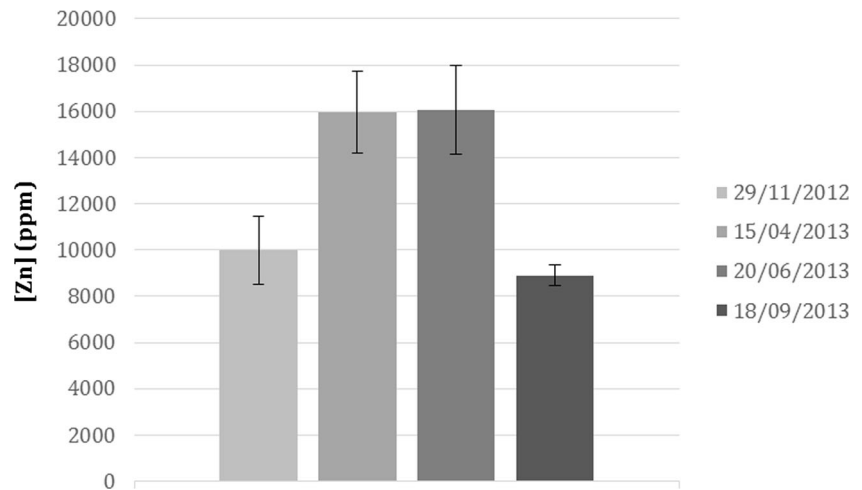
Fig. 4 Zinc concentration in *A. vulneraria* shoots by AAS method—60, 180, 240, and 300 days after transplanting date

As metallicolous *A. vulneraria*, many legumes are known to tolerate metals and grow in soils with high metal concentrations (Vara Prasad and de Oliveira Freitas 2003; L'Huillier et al. 2010). According to Pajuelo et al. (2007), legumes are not hyperaccumulators. However, a very few exceptions are found; legumes can hyperaccumulate heavy metals as Pb (Sahi et al. 2002) and other heteroatoms as Se (Alford et al. 2012). Therefore, the potential accumulation of *A. vulneraria* subsp. *carpatica* has been investigated over 5 years of harvest and over 500 plants (Fig. 3). It was compared for reference, with the well-known Zn-hyperaccumulator, *N. caerulescens*, also naturally present on the Les Avinières site.

The accumulation abilities for Zn, Cd, and Pb of *A. vulneraria* and *N. caerulescens* were studied using two independent methods for more reliability: atomic absorption spectrometry (AAS) and polarography.

As shown in Fig. 3, Zn concentration of 10,000 ppm threshold was reached (Mahmood 2010; Losfeld et al. 2012a); both plants could be considered as Zn-hyperaccumulator. The Zn concentration in shoots of *A. vulneraria* was even higher than in *N. caerulescens* shoots. Therefore, regarding the definition of phytostabilisation (Wong 2003; Padmavathiamma and Li 2007), using

A. vulneraria for such soil remediation is inappropriate. We propose herein using *A. vulneraria* for phytoextraction of a shortly depleted heavy metal, Zn.

Moreover, *A. vulneraria* appeared to be more metal selective than *N. caerulescens* regarding Cd accumulation.

Facing the high hyperaccumulation ability of *A. vulneraria* collected from Les Avinières, cultures of *A. vulneraria* were performed under controlled conditions in greenhouse to investigate the Zn accumulation profile. Zn accumulation in shoots was measured by AAS versus the elapsed time after transplantation; results are presented in Fig. 4.

The maximum Zn concentration, 17,428 ppm, was measured 240 days after transplanting and highly exceeded the Zn-hyperaccumulation threshold. Therefore *A. vulneraria* shoots were collected for biomass recovering 240 days after transplanting to get the highest yield of Zn extraction.

The *A. vulneraria* multi-symbiosis with rhizobia-like bacteria

As a legume, *A. vulneraria* establishes a symbiotic interaction with rhizobia-like bacteria, allowing it to grow in poor soils devoid of nitrogen nutriments. In 2009, Vidal et al. have identified a new symbiotic bacteria, *Mesorhizobium metallidurans*, of *A. vulneraria* subsp. *carpatica* from the mining site of Les Avinières. In 2013, Mahieu et al. have shown that *A. vulneraria* releases around 400 kg/ha of nitrogen in soil through rhizodeposition and decomposition of senescent tissue after 2 years of growth which increase soil fertility. Further investigations were performed on *A. vulneraria* symbiotic system, and more recently, a second symbiont has been found in its nodules, *Rhizobium metallidurans* (Grison et al. 2014a).
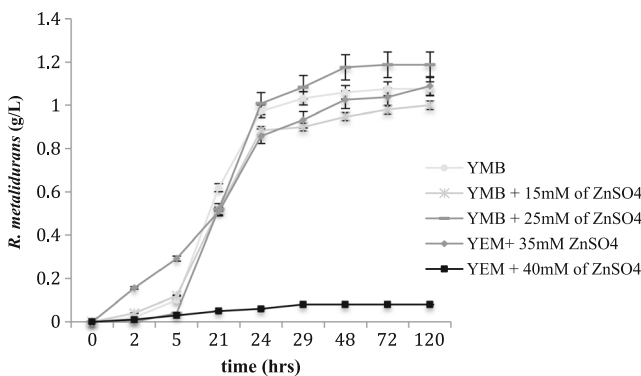

Fig. 5 Kinetic growth of *R. metallidurans* in artificially enriched YMB with ZnSO₄

Table 1 Specific growth value according to the ZnSO₄ concentration

YMB+[ZnSO₄]	0 mM	15 mM	25 mM	35 mM	40 mM
Specific growth (h⁻¹)	0.177	0.161	0.169	0.174	0.029

Table 2 Mineral composition of the Eco-Zn catalysts established by ICP-MS

Catalyst	Concentration (ppm)									
	Mg	Al	Ca	Mn	Fe	Ni	Cu	Zn	Cd	Pb
N. caerulescens Eco-Zn	15,921	10,904	100,641	594	48,322	584	409	**73,443**	**4,475**	**20,320**
A. vulneraria Eco-Zn	35,876	6,015	668,579	862	14,888	2,734	2,460	**154,202**	**533**	**11,700**

The values written in bold are the three key metals for catalysis

A. vulneraria subsp. *carpatica* presented a rare case of multi-symbiosis. The phenomenon was noted especially when microbial biodiversity is low; a hundred times less, microbial species was found in such a polluted soil. Vegetal diversity being also restrained in the mining site, *A. vulneraria* was the only legume present and available that could be infected by rhizobia. The multi-symbiosis could be considered as a form of adaptation to extreme conditions through a co-evolution between *A. vulneraria* subsp. *carpatica* and *M. metallidurans* and *R. metallidurans*.

Facing the objective of using *A. vulneraria* shoots as an ecocatalysis in the synthesis of precursors for chemical industry, the fast growth and abundance of starting material is required. Rhizobia living in symbiosis with *A. vulneraria* play here an interesting role. The symbionts promote the plant growth by producing ammonium from atmospheric N_2 fixation. Thereby a cost-efficient method to selectively culture them at a large scale is needed.

M. metallidurans and *R metallidurans* are tolerant to high concentration of heavy metals. Their tolerance has been tested in Cd- and Zn-enriched YEM solid media. On such media, the level of heavy metal is so high that only tolerant strains are able to grow. Addition of heavy metals in the culture medium can be therefore considered as a new cost-efficient method of selection like usually antibiotics.

Large-scale culture needs to be performed in liquid medium. *R. metallidurans* growth was tested in a wide range of YMB liquid media enriched with $ZnSO_4$ at 0, 15, 25, 35, and 40 mM (Fig. 5). Bacterial population evolution was determined by dry mass measurement since *R. metallidurans* was aggregating in liquid medium disturbing the OD measurement because of the production of extracellular polysaccharides (Dorken et al. 2012).

In Fig. 5, up to 35 mM of zinc, bacterial dry mass increased with time. *R. metallidurans* growth was not disturbed by such concentrations of $ZnSO_4$. As shown in Table 1, the specific growth rates during the exponential growth were all similar and confirmed that zinc, in a certain limit, had no impact on the strain growth and on the kinetic growth. Therefore, YMB medium enriched with 35 mM of $ZnSO_4$ could be optimum for industrial Rhizobia culture without affecting the kinetic growth and thus productivity.

The impact of the symbionts has been investigated on Zn extraction. When *A. vulneraria* was inoculated by *R. metallidurans*, the concentration of Zn in shoots was increased by 36 %. For large-scale phytoextraction in the pilot site of Les Avinières, *A. vulneraria* was then always inoculated by *R. metallidurans* to get the highest concentration of Zn.

An opportunity for Zn phytoextraction and biomass recovering in EcoCatalysis

Estimation of the biomass production per hectare could be made by calculating how many plants could cover 1 ha. The agronomic aspects of phytoextraction have been the subject of detailed calculations and modelling by Robinson et al. (2003). In its simplest form, the actual amount of zinc gained per hectare per annum in a phytoextraction process depends essentially on the proportional relationship between zinc concentration in the phytoextraction crop and its harvestable biomass yield, thus:

$$YNi = FNi.Ybio$$

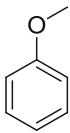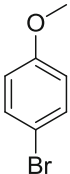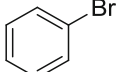FNi   Average fraction of zinc in *A. vulneraria* biomass
Ybio   Biomass yield of hyperaccumulator (kg/(ha/year))
YNi   Total zinc gain (kg/(ha/year))
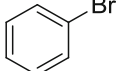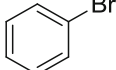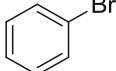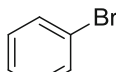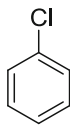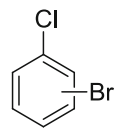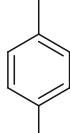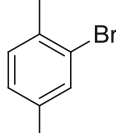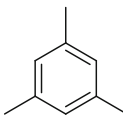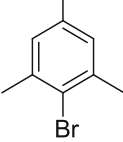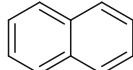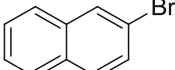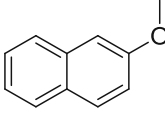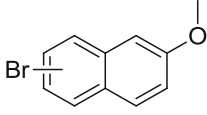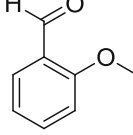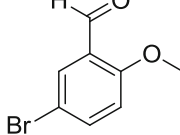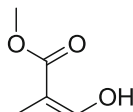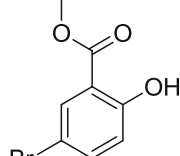
Assuming 70 % ground cover (Robinson et al. 2003), the number of *A. vulneraria* plant per hectare was estimated at 266,160 in the site of Les Avinières. The theoretical yield per hectare was 1791 kg/ha of dry biomass. The average fraction of zinc in *A. vulneraria* biomass, estimated at 1.5 % enabled us to calculate the total zinc gain: shoots from a crop of *A. vulneraria* could potentially remove 27 kg/ha of Zn.

This result has been compared with the Zn-hyperaccumaltor reference, *N. caerulescens*. The theoretical

Table 3 Zn/Cd and Zn/Pb ratios of the Eco-Zn catalysts

Catalyst	Ratio	
	Zn/Cd	Zn/Pb
N. caerulescens Eco-Zn	16.4	3.6
A. vulneraria Eco-Zn	**289.3**	**13.2**

The values written in bold are the three key metals for catalysis

Table 4 Bromination of aromatic substrates catalyzed by Eco-Zn catalyst compared to FeBr₃ and pyridine

Entry	Substrate	Product	Time	Temperature	Catalyst	Yield
1			6 h	25°C	Eco-Zn /K10	100 %
2			3 h	60°C	Eco-Zn /K10	100 %
3			3 h	60°C	FeBr₃	60 % (Vogel et al. 1996)
4			1 h then 45 min	30°C then 70°C	pyridine	60 % (Vogel et al. 1996)
5			3 h	60°C	K10	27 %
6			3 h	60°C	-	<1 %
7			6 h	60°C	Eco-Zn /K10	100 %
8			3 h	60°C	Eco-Zn /K10	100 %
9			3 h	60°C	Eco-Zn /K10	100 %
10			3 h	40°C	Eco-Zn /K10	100 %
11			17 h	25°C	Eco-Zn /K10	83 %
12			3 h	40°C	Eco-Zn /K10	100 %
13			3 h	60°C	Eco-Zn /K10	100 %
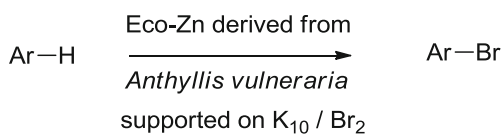
Scheme 1 General bromination of aromatic substrates catalyzed by Eco-Zn catalyst

Scheme 2 Extension to the bromination of thiophene

yield per hectare was 1632 kg/ha and the average fraction of zinc in *N. caerulescens* was 1 %. Shoots from a crop of *N. caerulescens* could only remove 16.8 kg/ha of Zn.

Efficient recovering of *A. vulneraria*-contaminated shoots

In phytoextraction, contaminated shoots of extracting plants are too often considered as wastes and are poorly valorised. An efficient method to recover Zn from *A. vulneraria* shoots is described here through the preparation of heterogeneous EcoCatalysts used in synthetic transformations.

The selection of conditions guided us towards a simple, low-cost, and efficient process in accord with the principles of green chemistry (Anastas and Warner 1998). It is also compliant with direct industrial applications. Two parameters were crucial: a high concentration of Zn in leaves promoted an efficient catalysis and a high ratio of Zn/Cd and Zn/Pb limited the amount of Cd and Pb in the catalytic structure. This aspect was crucial because these last elements were not eco-friendly elements.

A comparison between both Zn-hyperaccumulating plants from Les Avinieres, *A. vulneraria* and *N. caerulescens*, was required. Motivated by the chemical recovery of these plants, the study was performed after the transformation of their biomass into Eco-Zn catalysts. Biomass was dried and crushed. A thermal treatment was then applied to destroy organic matter. The ashes obtained were treated with hydrochloric acid to finish the destruction of the remaining organic compounds and to convert metallic cations into metal chlorides. The resulting solutions, composed of different metal chlorides, were then concentrated and dried to obtain Eco-Zn catalysts. Inductively coupled plasma-mass spectrometry (ICP-MS) analyses were used to determine the composition of the Eco-Zn catalysts obtained. Table 2 exemplifies the composition of the different plant extracts obtained.

The Zn level of Eco-Zn from *A. vulneraria* was higher than Zn level of Eco-Zn from *N. caerulescens* as noticed in shoot concentration. The metal hyperaccumulation selectivity was
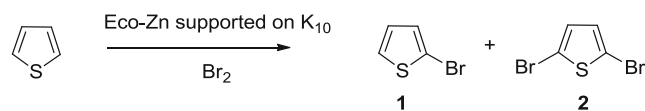
investigated by measuring the ratios of Zn/Cd and Zn/Pb, summarized in Table 3.

The difference between the two systems was spectacular. *A. vulneraria* phytoextracted Zn much more selectively than *N. caerulescens* and so led to a higher concentration of Zn in the ecocatalyst.

A. vulneraria represents a very good metallophyte—Zn concentration was maximized in shoots; non-eco-friendly elements (Pb and Cd) concentrations were very low; and biomass production was high and led to high total gain of Zn. It represents an important result for the chemical perspective but also on a theoretical level: it shows the advantage of *A. vulneraria* for phytoextraction programs.

Encouraged by these results, we explored the chemical recovery of biomass produced by *A. vulneraria*, *A. vulneraria* Eco-Zn, through a reaction of industrial interest, the bromination of aromatic susbstrates.

### Application in ecocatalysis bromination via electrophilic aromatic substitution
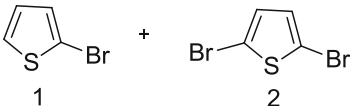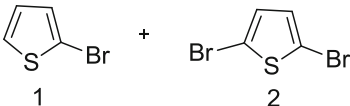
Brominated aromatic compounds are widely used by the chemical industry, in benzene, heterocyclic, polycyclic series. These compounds are used as precursors for the synthesis of molecules of economic interest, such as drugs (e.g., nicergoline, bromocriptine, brotizolam), colors (e.g., 6,6′-dibromoindigo), flame retardants (e.g., tetrabromobisphenol A), and colored indicators (e.g., bromothymol blue) (see Table 4).

The common method to introduce a *bromo atome* on an aromatic ring is the electrophilic aromatic substitution (EAS). Bromine itself is not electrophilic enough to react with benzene. The introduction of a catalyst is essential to lead to the substitution product. It can be catalyzed by $FeBr_3$, used as a Lewis acid or by pyridine, which activates bromine electrophily (Vogel et al. 1996). However, bromination catalyzed by $FeBr_3$ or pyridine is not completed and often leads to a mixture of mono- and dibromo-aromatic product (Kalsi 2007).

Experiments were performed to examine bromination with Eco-Zn green system in heterogenous catalysis. The general EAS reaction is described in Scheme 1 and has been applied to a range of examples in Table 4.

A catalytic system was obviously needed for the bromination of aromatic substrates (entries 5 and 6 in Table 4). The catalysts developed from Eco-Zn allowed bromination by electrophilic substitution of many aromatic compounds

Table 5 Sheldon's E factor for benzene bromination

Catalytic system	E factor
$FeBr_3$	5.76
Pyridine	3.04
Eco-Zn	1.20

Table 6 Regioselectivity of bromination reaction on thiophene

Substrate	Product	Time	Temperature	Yield
			°C	(ratio 1/2)
	1 + **2**	1 h 30	25	1 : 45 % 2 : 25 % (1/2 = 1.8)
	1 + **2**	2 h	0	1 : 36 % 2 : 8 % (1/2 = 4.6)

(entries 1, 2, and 7–13). The reactions catalyzed by Eco-Zn were rapid and selective and led to high yields. Eco-Zn (entry 2) was more efficient than FeBr$_3$ (entry 3). The reaction was carried out without solvent other than the brominated substrate, when liquid at the reaction temperature. Bromination was realizable with a wide range of methoxy, methyl, hydrogen, chloro, formyl, and methylcarboxy substituents and with mono or polyaromatic substrates. The steric hindrance did not modify the efficiency of EAS and no polybromination was observed. The ecocatalyst was reused and retained optimum activity until four cycles; after completion of the reaction, the supported catalyst was filtered and dried easily by heating at 110 °C for 30 min.

A comparison between FeBr$_3$, pyridine (Vogel et al. 1996) and Eco-Zn was demonstrative. The three catalytic systems were studied through benzene bromination and compared with Sheldon's E factor (Sheldon 2007; Sheldon 2008) (Table 5).

Eco-Zn clearly led to the lowest E-factor compared to classical methods. No further purification was needed; a simple filtration was readily performed to recover the active Eco-Zn. On the contrary, when using both conventional catalysts, aqueous treatment was required and led to wastes containing toxic compounds. A non-negligible quantity (16 %) of side product, *para*-dibromobenzene, was also obtained and required further purification.

Particular interest lies in using Eco-Zn catalyst derived from *A. vulneraria* in bromination of heterocycles. Thiophene is widely used by chemical industry as an interesting starting material in EAS; it has been the object of a particular study (Scheme 2).

Under the conditions described in Table 6, Eco-Zn catalyst was able to catalyze bromination of thiophene with satisfactory yields. Classically, the procedure for the bromination of thiophene consists of adding an equimolar amount of bromine to thiophene while cooling. However, the 2,5-dibromothiophene is predominant. Only a minor amount of monobromine was obtained here. Pleasingly, the reaction catalyzed by Eco-Zn allowed a good control of monobromination.

Conclusion

A. vulneraria had been used in phytoremediation process, in phytostabilisation so far. However, *A. vulneraria subsp. carpatica* was able to translocate and hyperaccumulate very high concentration of Zn in shoots. A maximum of 17,428 ppm of Zn was reached in shoots which clearly exceeded the hyperaccumulation threshold of 10,000 ppm and the Zn concentration of a Zn-hyperaccumulator reference, *Noccea caerulescens*. Total gain of Zn of *A. vulneraria* was 38 % higher than the one of *N. cearulescens*. Therefore, the huge hyperaccumulation abilities and the large biomass production conducted to develop a phytoextraction process with *A. vulneraria subsp. carpatica*. The phytoextraction of Zn was optimized by inoculating *A. vulneraria* with *R. metallidurans*, a natural symbiont. Zn concentration in shoots was increased by 36 % after inoculation. Selective culture of *R. metallidurans* was investigated in a large scale in liquid medium, artificially enriched with 35 mM of ZnSO$_4$ for the development of the phytoextraction process on a pilot site. Contaminated shoots of *A. vulneraria* were not considered as wastes and were recovered as Eco-Zn catalyst in electrophilic aromatic substitution. Eco-Zn catalyst led to much higher yields and deeply lower Sheldon's E-factor than conventional catalysts. Eco-Zn catalyst derived from contaminated shoots of *A. vulneraria subsp. carpatica* consisted

of a new generation of green catalyst by its vegetal source and by its catalyst activity in organic synthesis.

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