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ECOLOGY AND CONSERVATION OF BELGIAN POPULATIONS OF *VIOLA CALAMINARIA*, A METALLOPHYTE WITH A RESTRICTED GEOGRAPHIC DISTRIBUTION

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ABSTRACT. — *Viola calaminaria* (Gingins) Lej. is a rare and threatened species, endemic to metalliferous soils in E Belgium, S Netherlands and W Germany. In order to provide basic information for a conservation strategy, we performed an ecogeographic survey of almost all *V. calaminaria* populations currently existing in Belgium. Twenty-four populations were found, distributed in three geographic groups all in the Province of Liège. The area covered by *V. calaminaria* ranged from < 1 m^2 to 3.2 ha. The largest populations were found in sites contaminated by atmospheric deposits from metal smelters. Soils were extremely variable in heavy metal and nutrient concentrations, but concentrations of Zn, Pb and Cd were consistently higher than reference values for normal soils. *V. calaminaria* was most often found in association with other metallophyte and pseudometallophyte taxa typical of metalliferous sites. Plant communities identified by TWINSPAN analysis generally fitted the heavy metal associations previously described but two unusual heavy metal plant communities were identified. Based on a canonical correspondence analysis, pH was the only factor that was clearly correlated with the floristic composition of plant communities associated to *V. calaminaria*. This study confirmed the ecological endemic status of *V. calaminaria* and the importance of the conservation of metalliferous sites.

KEY WORDS. — *Viola calaminaria*, rare species, heavy metals, ecogeography, conservation.

INTRODUCTION

A taxon can be considered as rare when it meets at least one or a combination of the following criteria : 1) restricted geographic distribution, 2) habitat with restricted ecological conditions, 3) small population size (OLIVIERI & VITALIS 2001). Rarity in itself is not synonymous with extinction threats (DE LANGHE & NORTON 2004), but because of their limited geographical and ecological extension, rare endemic taxa are more prone to be driven to extinction by anthropogenic habitat destruction. While rare endemic taxa are often adapted to scattered small habitats, habitat fragmentation may increase the risk of local population extinction due to demographic stochasticity, genetic drift and inbreeding (PRIMACK 1998, OLIVIERI & VITALIS 2001). For these reasons, rare endemic taxa are considered as priority targets for biodiversity conservation programmes, in particular when threats on their habitat can be clearly identified (SCHEMSKE *et al.* 1994, LOVETT DOUST & LOVETT DOUST 1995, PRIMACK 1998). As a first approach, the conservation of rare endemic taxa requires an investigation into their biogeography, including geographic distribution, niche characterisation and particularly the limiting environmental factors and the impact of human factors, both past and present (DEBUSSCHE *et al.* 1996, DINSDALE *et al.* 1997, MAXTED 1999, DE LANGHE & NORTON 2004).

Soils with high concentrations of heavy metals (metalliferous soils) are a good example of scattered habitat. Because of their phytotoxicity, metalliferous soils represent very restrictive habitats for plants (ANTONOVICS *et al.* 1971, ERNST 1990, MACNAIR & BAKER 1994, BROWN 2001). In addition, metalliferous sites are generally of small size and geographically isolated from each other within a landscape matrix with normal background metal concentrations (but see WOLF 2001 for a discussion of scale effect). These sites provide outstanding examples of micro-evolution and speciation processes due, on the one hand, to the severe selection pressure induced by heavy metals and, on the other hand, to founder effects and genetic drift induced by the insularity of these habitats (ERNST 1990, LEFÈBVRE & VERNET 1990, MENGONI *et al.* 2000). As a result, metalliferous sites often host rare, ecologically endemic taxa adapted to high levels of heavy metals. In Belgium, metalliferous sites consist of calamine soils exhibiting high concentrations of zinc, cadmium and lead (LAMBINON & AUQUIER 1963, SIMON 1978, DUVIGNEAUD 1982, DUVIGNEAUD *et al.* 1993, DUVIGNEAUD & SAINTENOY-SIMON 1996). These sites host a number of metallophytes at various taxonomic ranks (DUVIGNEAUD 1982, DUVIGNEAUD *et al.* 1993, DUVIGNEAUD & SAINTENOY-SIMON 1998).

Of all these taxa, *Viola calaminaria* (Gingins) Lej. is of particular interest. *Viola calaminaria* was first described by LEJEUNE (1811). It was initially regarded as a variety (*Viola lutea* Huds. var. *multicaulis* Koch ; LEJEUNE 1811) or as a subspecies of *Viola lutea* Huds. (*Viola lutea* Huds. subsp. *calaminaria* (Gingins) Nauenburg ; NAUENBURG 1986) on the basis of morphological characters. More recently, on the basis of cytological criteria, it was considered as a true species derived from *Viola tricolor* L. subsp. *subalpina* Gaud. by polyploidisation during the ice age (HEIMANS 1961, KAKES 1979). Ernst (cited in KAKES & EVERARDS 1976) argued that *V. calaminaria* recently derived from *Viola tricolor* L. while ROSTANSKI (2003) argued that *V. calaminaria* is not distinct from *Viola lutea.* According to ERNST (1990) and KAKES (1977), two subspecies should be recognised within *V. calaminaria* : *Viola calaminaria* subsp. *calaminaria* Lej. and *Viola calaminaria* subsp. *westfalica* (Lej.) Ernst, the latter with purple flowers being restricted to W Germany. Other authors considered them as two distinct species : *Viola calaminaria* and *Viola guestphalica* Nauenburg (NAUENBURG 1986, HILLDEBRANDT *et al.* 1999, ROSTANSKI 2003). In Belgium, *V. calaminaria* is considered as a rare calamine-endemic species (LAMBINON 1992). Undoubtedly, whatever its taxonomic position, *V. calaminaria* represents an original genetic pool differentiated from related species and is worth of conservation effort because of its ecological particularity.

Calamine sites are threatened in Belgium. They are often considered as waste ground dangerous for human health and for which public authorities are inclined to promote site remediation. Several sites have disappeared over the past decades and others have been partially altered by mining, grazing, road or public equipment building and recreational activities (DUVIGNEAUD 1982, GRAITSON 2004). This pressure has led to habitat loss and fragmentation, decrease in number and size of remaining metallophyte populations and consequently potential problems of population and metapopulation viability for metallophyte taxa.

Current knowledge of *V. calaminaria* ecology and distribution is still incomplete. This was illustrated by the recent discovery of previously overlooked populations (DUVIGNEAUD 1976, JORTAY 1984, GRAITSON 2004). Up to now, no study has specifically assessed the ecological niche of the species. In particular, the range of high heavy metal concentrations required for the development of *V. calaminaria* populations is not known. The aim of this study was to assess the distribution and ecological amplitude of *V. calaminaria* in Belgium in order to provide the basis for the definition of a conservation strategy. Specific aims were : i) to examine the current distribution of the species and evaluate the size of the populations, ii) to assess the ecological amplitude of the species in relation to heavy metals in soil, and iii) to study the associated flora and vegetation of *V. calaminaria* populations.

MATERIAL AND METHODS

PLANT SPECIES

V. calaminaria is a perennial clonal pansy with a prostrate habit (8 to 40 cm). Vegetative propagation is achieved by means of long rhizomes and stolons. The species flowers from May to October with the flowering peak between May and July. Flowers are generally yellow but some individuals exhibit light to dark blue petals (LAMBINON 1992). The species distribution includes the east of Belgium (north east of the Liège province), the south east of Dutch Limburg and the vicinity of Aachen (west of Germany).

POPULATION SURVEY

Historical records of *V. calaminaria* in the Walloon Region (South Belgium) have been documented on the basis of a review of the specialised naturalist and scientific literature (e.g., LAMBINON & AUQUIER 1963, DUVIGNEAUD 1982, DUVIGNEAUD & JORTAY 1987, DUVIGNEAUD *et al.* 1993, FAGOT 1993, DUVIGNEAUD & SAINTENOY-SIMON 1996, BECKERS 1997) and on the recent synthesis by GRAITSON (2004). All sites with historical or recent reports of *V. calaminaria* populations were visited in the spring of 2003 to check for the presence of the species. The size of *V. calaminaria* populations was estimated. Because of the clonal habit of the species, we were not able to census the number of individuals (i.e., genets). *V. calaminaria* is generally distributed in patches of various densities. The size of each population was then evaluated as the surface area of covered by the species on the basis of a combination of two parameters : the total surface of patches in the site and the percentage of ground cover of the species in these patches. The outlines of patches were delimited by GPS. Patch area was calculated with the XTOOLS ARC VIEW extension. The percentage of *V. calaminaria* groundcover was estimated for each patch on the basis of the linear cover of the species. To that end, the cover of the aerial parts of the species was measured (precision : 5 cm) along a graduated line randomly located within each patch. The percentage of groundcover was estimated as the ratio between the total length covered by *V. calaminaria* (m) and the total length of the transect (m). When *V. calaminaria* was present in different plant communities, the percentage of cover was evaluated separately for each of them. The two measures (percentage of cover within patches and total area of patches) were multiplied to obtain a population area estimate used as a surrogate of population size.

ECOLOGICAL AMPLITUDE OF THE SPECIES

Population sampling

As one of the aims of the study was to make a synthesis of the ecological amplitude of *V. calaminaria* in regard to the recent discovery of new populations, floristic and soil analyses focused on populations for which no ecological data could be found in the literature. Sampled sites were assigned to one of the following categories : i) primary sites that are natural mineral outcrops (I), ii) secondary sites in place that result from mining activities in primary sites (IIa), iii) industrial secondary sites that consist of furnace slag and cinders resulting from industrial activity (IIb) and iv) tertiary sites resulting from atmospheric pollution (III). Thirteen sites were selected to be representative of the different origins and geographical regions. At each site, a stratified sampling strategy was used to ensure that the full ecological variation of the populations was described. On a brief reconnaissance, the surfaces where *V. calaminaria* was present were divided into major vegetation types on the basis of the vegetation structure and dominant species (facies). Sampling quadrats for floristic relevés and soil sampling (same quadrats) were located in the different major communities associated to *V. calaminaria* within each site.

Associated flora and plant communities

In each site, three floristic relevés (1.5 m × 1.5 m quadrats) were performed in each vegetation facies

TABLE 1

List of sites hosting V. calaminaria *with localisation (commune and x, y Lambert coordinates), altitude, total area, type of site,* V. calaminaria *population size estimates (patch size multiplied by % cover),* TWINSPAN *group classification (only for sites included in the phytosociological study) and site localisation of relevés (only for sites for which soil samples were analysed)*

Name	Commune	X_center Lambert coordinate	Y_center Lambert coordinate	Altitude (m)	Site area (ha)	Type of site	Population size (m^2)	TWINSPAN groups	Site localisation of relevés (Rx)
Ancien jumping de Chaudfontaine	Chaudfontaine	241041	142500	80	4,11	III	1769		
Bois-les-Dames	Chaudfontaine	240835	141864	200	51,68	III	4590	G7	R1, R2, R4
Fonds-de-Forêt	Trooz	242837	142164	115	6,64	III	1651		
Prayon	Trooz	242333	142372	160	51,97	III/IIb	32242	G7	R19, R22, R25
Jardin à Prayon	Trooz	241614	141931	80	0,11	III	0,002		
Ile aux Corsaires	Liège	237854	145398	60	3,71	IIb/III	3587		
Friche industrielle à Angleur	Liège	237771	145264	60	3,86	IIb/III	1517	G6	R7, R8, R10
Petit remblai à Angleur	Liège	237931	145106	65	0,07	IIb/III	19		
Parc de la Vieille Montagne	Liège	237826	144819	65	7,78	III	4898		
Voie ferrée et gare à Chênée	Liège	238042	145007	70	0,96	III	304		
Sur-les-Tiers	Liège	237924	145896	110	2,21	III	3744	G7	R13, R14, R15
Lande de Streupas	Liège	237184	144599	160	16,67	III	219	G2	
Halde du Casino	Kelmis/ La Calamine	265849	156835	180	2,75	IIb	36	G1	
Réserve de La Calamine	Kelmis/ La Calamine	265920	157253	190	0,37	I	0,4	G4	R34, R35, R36
Schmalgraf	Kelmis/ La Calamine	264867	155173	190	0,9	IIa	150	G2, G3	
Petite halde du Schmalgraf	Kelmis/ La Calamine	264357	155331	233	0,05	IIb	60	G3, G6	R46, R47, R48
Pelouse près de la ferme Huset	Kelmis/ La Calamine	265130	155293	210	0,07	I	0,002	G5	R49, R50, R51
Rabotrath	Lontzen	266390	152285	260	1,67	IIa	60	G3, G4	R52, R53, R54
Plombières	Plombières	262974	159322	155	22,82	IIa/IIb	5815		
Terbruggen	Plombières	261083	161234	135	0,16	IIb	0,06	G1	R55, R56, R57
Ru de Wilcour	Welkenraedt	262126	151273	260	0,39	IIa	145		
Le Rocheux	Theux	253651	137567	230	3,47	IIa	25	G2, G4	R58, R59, R61
Petit tumulus face au Rocheux (Sud)	Theux	253425	138072	225	0,06	IIb	51		

using the Braun-Blanquet scale. Three to nine relevés were thus obtained in each site for a total of 63 relevés (see Table 1 for identification of sites included in the vegetation analysis). Nomenclature followed LAMBINON *et al.* (1992). Vegetation data were analysed using two-way indicator species analysis (TWINSPAN ; HILL 1979).

Soil analysis

Ten out of the thirteen selected sites were retained for soil analysis (sites for which no data existed in the literature, see Table 1). Three bulk samples were collected at each site (at least one sample per facies) for a total of 30 samples. All samples were collected within the quadrats used for the vegetation relevés. In two quadrats (Terbrugen site), *V. calaminaria* was not present. Each sample consisted of five bulked subsamples (depth 0-15 cm, organic layer excluded) collected at the four corners and the center of the quadrat. Samples were air-dried, sieved to 2 mm, and weighted before and after sieving. The proportion of stones was determined as the ratio of the weight of particles larger than 2 mm to the total dry weight of the sample. pH_{H_2O} was measured for each sample with a digital pH-meter. Cations were extracted with 1N ammonium acetate-EDTA (pH 4.65) by the Cottenie method (COTTENIE *et al.* 1975). Ca, Zn, Cd, Pb concentrations were determined by ICP-OES. Pb/Ca and Zn/Ca ratios were calculated as they better reflect actual metallic toxicity than absolute metal concentrations (SIMON 1978, GARLAND & WILKINS 1981, BROWN 2001).

Statistical analyses were performed with XLSTAT version 6.0 and MINITAB version 13.20. Relationships between floristic data and soil parameters were examined by a Canonical Correspondence Analysis (CCA) using CANOCO for WINDOWS version 4.5 (TER BRAAK & SMILAUER 2002).

RESULTS

POPULATION SURVEY

A total of twenty-three populations were found (Fig. 1a, Table 1). Some of the observed populations were very close to each other (less than 50 m) and may thus represent subpopulations from a genetic point of view. Nevertheless, as our survey should be useful for a conservation strategy, we considered that groups of individuals separated spatially from other groups of individuals and situated in sites with different land occupation were different populations, each one of which forming a distinct management unit.

All populations were located in the Province of Liège. Three geographic groups could be identified (Fig. 1b) : 13 populations in the vicinity of Liège, two populations in the vicinity of Theux and nine populations in the region of Kelmis.

The cumulated size of all populations was 6.09 ha. The size of individual populations varied greatly (Table 1, Fig. 2). Four populations were extremely small (< 1 m^2), six populations displayed a small size (between 1 and 100 m^2), 12 populations displayed a moderately large size (from 100 m^2 to 1 ha) and the largest one reached over 3.22 ha. The single largest population (Prayon) represented 53% of the total *V. calaminaria* area. A significant positive correlation was found between the total site area and the size of the area occupied by *V. calaminaria* in each site (Spearman rank correlation, $r^2 = 0.64$, $P < 0.001$). The correlation was still significant when the largest site with the largest population (Prayon) was removed (Spearman rank correlation, $r^2 = 0.60$, $P < 0.001$).

ASSOCIATED FLORA AND PLANT COMMUNITIES

The classification groups resulting from the TWINSPAN analysis are shown in Table 2. Only characteristic species (indicator and preferential) identified by TWINSPAN are shown (31 species on a total of 54 across all relevés).

Seven groups of relevés were identified (G1-G7). The classification of the different sites in the groups of relevés is displayed in Table 1. Group G1 was composed of *Festuca* sp. associated with *Deschampsia cespitosa*, *Phragmites australis*, *Stellaria holostea*, *Silene vulgaris* subsp. *vulgaris* var. *humilis*, and *Polygala vulgaris*. *Deschampsia cespitosa* and *P. australis* indicated a relatively high water table. Group G2 contained *Molinia caerulea*, *Calluna vulgaris*, *Festuca ophioliticola* subsp. *calaminaria*, and *Cerastium fontanum* as character species associated with *Holcus lanatus*, *Luzula campestris*, and *Rumex acetosa*. The first two characteristic species indicated relatively acid conditions. Group G3 was composed of *Agrostis capillaris*, *Thlaspi caerulescens* subsp. *calaminare*, and *Festuca ophioliticola* subsp. *calaminaria* as characteristic taxa with *Molinia*

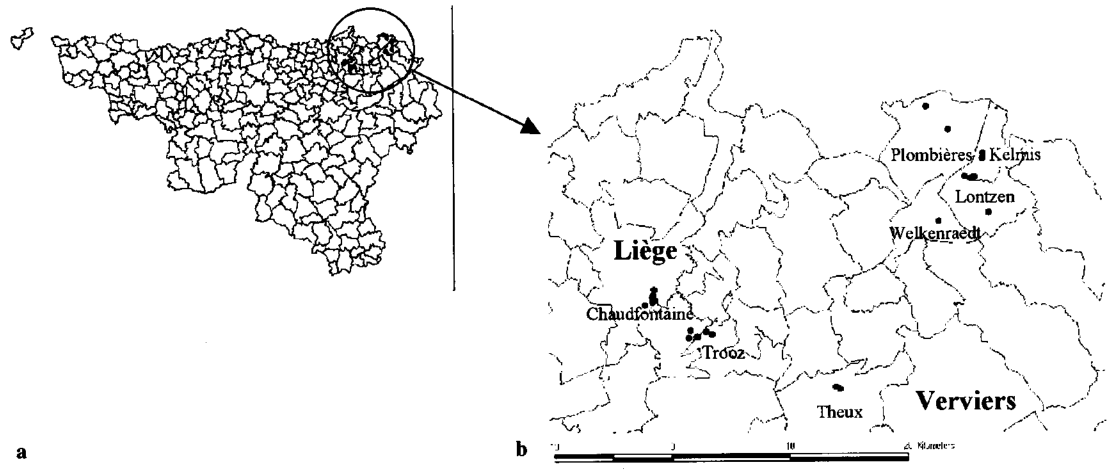

FIG. 1. — Localisation of *V. calaminaria* populations (represented by a black circle) in a) Wallonia, b) Eastern Wallonia.

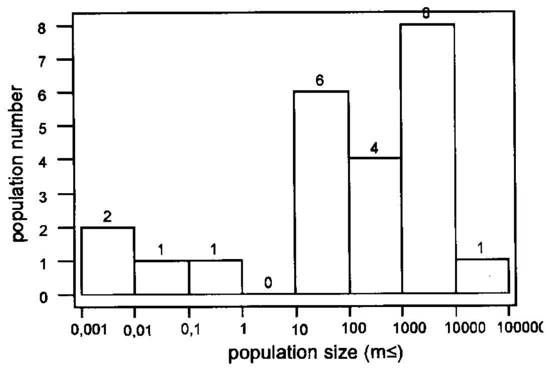

FIG. 2. — Frequency distribution of *V. calaminaria* population size expressed as the area covered by the species (m²). See text for details on surface estimation.

caerulea, *Plantago lanceolata*, and *Ranunculus acris*. Group G4 was characterised by *Thlaspi caerulescens* subsp. *calaminare*, *Armeria maritima* var. *halleri*, *Polygala vulgaris*, and *Fetusca ophioliticola* subsp. *calaminaria* associated with *Avenula pratensis*, *Minuartia verna* var. *hercynica*, *Rumex acetosa*, and *Silene vulgaris* subsp. *vulgaris* var. *humilis*. It was the group with the largest number of metallophytes. Group 5 comprised all relevés from a single site (Huset) and was characterised by *Galium mollugo*, *Ranunculus acris*, *Silene dioica*, and *Silene latifolia* subsp. *alba* associated with *Festuca* sp., *Lamium galeobdolon*, *Pimpinella saxifraga*, *Festuca ophioliticola* subsp. *calaminaria*, and *Stellaria holostea*. Most of these species were not found on the other sites and were characteristic of forest fringes on nutrient-rich soils. *Silene vulgaris* var. *humilis* and *Armeria maritima* var. *halleri* were characteristic taxa of group G6, associated with *Campanula rotundifolia*, *Carex spicata*, *Plantago lanceolata*, and *Arrhenatherum elatius*. Group G7 was characterised by *Agrostis capillaris* as character species associated with *Cerastium fontanum*, *Rumex acetosa*, and *Sonchus asper*.

SOIL PARAMETERS VARIABILITY

Soil parameters description

Soil pH was rather homogeneous, with most samples comprised between pH 6.0 and pH 7.0 (mean ± SD = 6.36 ± 0.75 ; Fig. 3b). *V. calaminaria* can thus be considered as a neutrophilous species. The proportion of soil particles larger than 2 mm varied from 1.3% to 53.5 % (mean ± SD = 26.9 ± 15.5 % ; Fig. 3a), indicating that the species can accommodate soils with very different structures.

The heavy metals content (Pb, Cd, Zn) was quite variable (up to three orders of magnitude ; Fig. 4 a-c). Nevertheless, these concentrations

TABLE 2

Key species (underlined) and preferential species of each classification group obtained with TWINSPAN *analysis*

Species	Classification groups						
	G 1	G 2	G 3	G 4	G 5	G 6	G 7
Viola calaminaria	IV	V	V	V	V	V	V
Ranunculus acris			III		V		
Plantago lanceolata			III			IV	
Silene vulgaris subsp. *vulgaris* var. *humilis*	III			II		V	
Agrostis capillaris			IV				IV
Cerastium fontanum		III					III
Molinia caerulea		IV	II				
Polygala vulgaris	II			IV			
Rumex acetosa				III			V
Armeria maritima var. *halleri*				IV		V	
Stellaria holostea	II				IV		
Deschampsia cespitosa	II						
Festuca sp. *(FES 1)*	V						
Phragmites australis	II						
Calluna vulgaris		III					
Holcus lanatus		II					
Luzula campestris		II					
Thlaspi caerulescens subsp. *calaminare*			II	V			
Avenula pratensis				II			
Minuartia verna var. *hercynica*				III			
Festuca ophioliticola subsp. *calaminaria*		V	V	V	II		
Festuca sp. *(FES 2)*					II		
Galium mollugo					II		
Lamium galeobdolon					II		
Silene latifolia subsp. *alba*					V		
Silene dioica					V		
Pimpinella saxifraga					II		
Campanula rotundifolia						II	
Carex spicata						II	
Sonchus asper							II
Arrhenatherum elatius						IV	
Number of relevés per group	9	9	9	8	3	7	18

Constancy coefficients : I, species present in less than 20 % of relevés ; II , species present in 20 % to 40 % of relevés ; III, species present in 40 % to 60 % of relevés ; IV, species present in 60 % to 80 % of relevés ; V, species present in 80 % to 100 % of relevés.

were always largely superior to heavy metal concentrations in normal soils and in most cases were superior to phytotoxicity levels. Zinc concentrations ranged from 105 mg kg^{-1} to 43619 mg kg^{-1} (mean ± SD = 11886 ± 13695 mg kg^{-1}) but most of the samples (83 %) had a concentration superior to 1000 mg kg^{-1}. Lead concentrations ranged from 85 mg kg^{-1} to 85329 mg kg^{-1} (mean ± SD = 9342 ± 19539 mg kg^{-1}) with most samples ranging from 100 mg kg^{-1} to 10000 mg kg^{-1}. Cadmium concentrations varied from 1.1 mg kg^{-1} to 117 mg kg^{-1} (mean ± SD = 24 ± 27 mg kg^{-1}). The sites Friche d'Angleur, Prayon, Huset, Rabotrath and Rocheux (see Table 1 for location) presented the highest concentrations in Zinc whereas for Pb the highest concentrations were observed in Huset, Rocheux and Friche d'Angleur. Pb/Ca and Zn/Ca ratios were highly variable. Pb/Ca (mean ± SD = 3.1 ± 3.7) was highest in Rocheux, Huset and Friche d'Angleur whereas Zn/Ca (mean ± SD

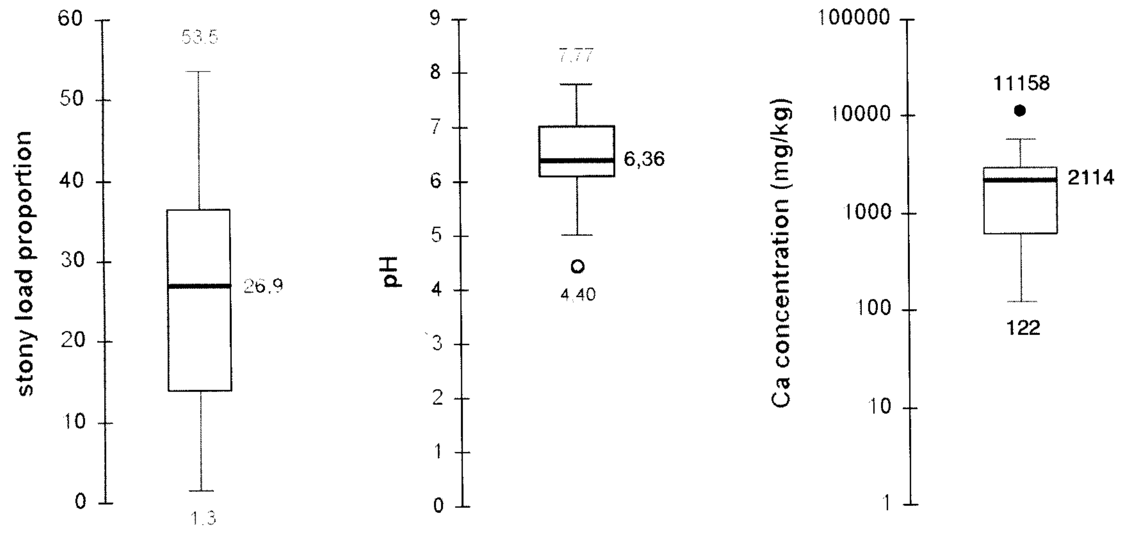

FIG. 3. — Variation of selected soils properties in ten populations of *V. calaminaria*. Box and whisker plots : a) stony load proportion (%), b) pH and c) Ca concentration (mg kg^{-1}).

= 5.8 ± 6.0) was highest in Rocheux, La Calamine, Prayon and Rabotrath (Fig. 4 d).

Mean values of soil parameters were compared between the different site categories (primary, secondary in place, industrial secondary and tertiary) with a two-way nested ANOVA with populations nested within site category. Two sites were considered as primary (Huset and La Calamine), two as secondary (IIa : Rocheux and Rabotrath), three as secondary (IIb : Friche d'Angleur, Terbruggen and Petite halde du Schmalgraf) and three as tertiary (Prayon, Bois les Dames and Sur le Tiers). Significant variations were found among populations within categories for Zn, Pb, Cd, Ca, Pb/Ca, Zn/Ca, pH, Mg. In contrast, no significant variation was found between the different site categories.

Influence of soil conditions on associated vegetation

In order to investigate the relationships between floristic variation and soil factors, data from quadrats with both floristic relevés and soil analyses (30 quadrats, 40 species and 9 soils parameters) were analysed by CCA. Fig. 5 displays the first two axes of a quadrat-environment biplot and a species-environment biplot with the three significant soil variables selected by a Monte-Carlo test. The eigenvalues were 0.338 for the first axis and 0.279 for the second axis. Together, these two axes explained 18.6 % of the floristic composition variation and 81.4 % of the species-environment relationship. Significant soil parameters were, by order of decreasing importance, Pb concentration ($P = 0.007$), pH_{H_2O} ($P < 0.001$) and Cd concentration ($P = 0.039$). Correlations between Pb and Cd concentration and axes were evenly split between the first two axes. Three relevés (R49, R50 and R51) from the same site were isolated in the quadrant of the biplot determined by Pb and Cd concentration. They formed a distinct group characterised by a particular species composition and corresponded to the G5 group in the TWINSPAN analysis (Table 2). pH was the variable most highly correlated with the second axis. This variable best explained the distribution of the other relevés. Relevés on acidic soil (R56, R57, R2, ...) were characterised by *Agrostis capilaris*, *Luzula campestris* and *Holcus lanatus* while those on neutral soil (R7, R8, R10, R54 ...) were characterised by *Campanula rotundifolia* or *Carex spicata*. Most metallophytes grouped together close to the centre of the plot : *V. calaminaria*,

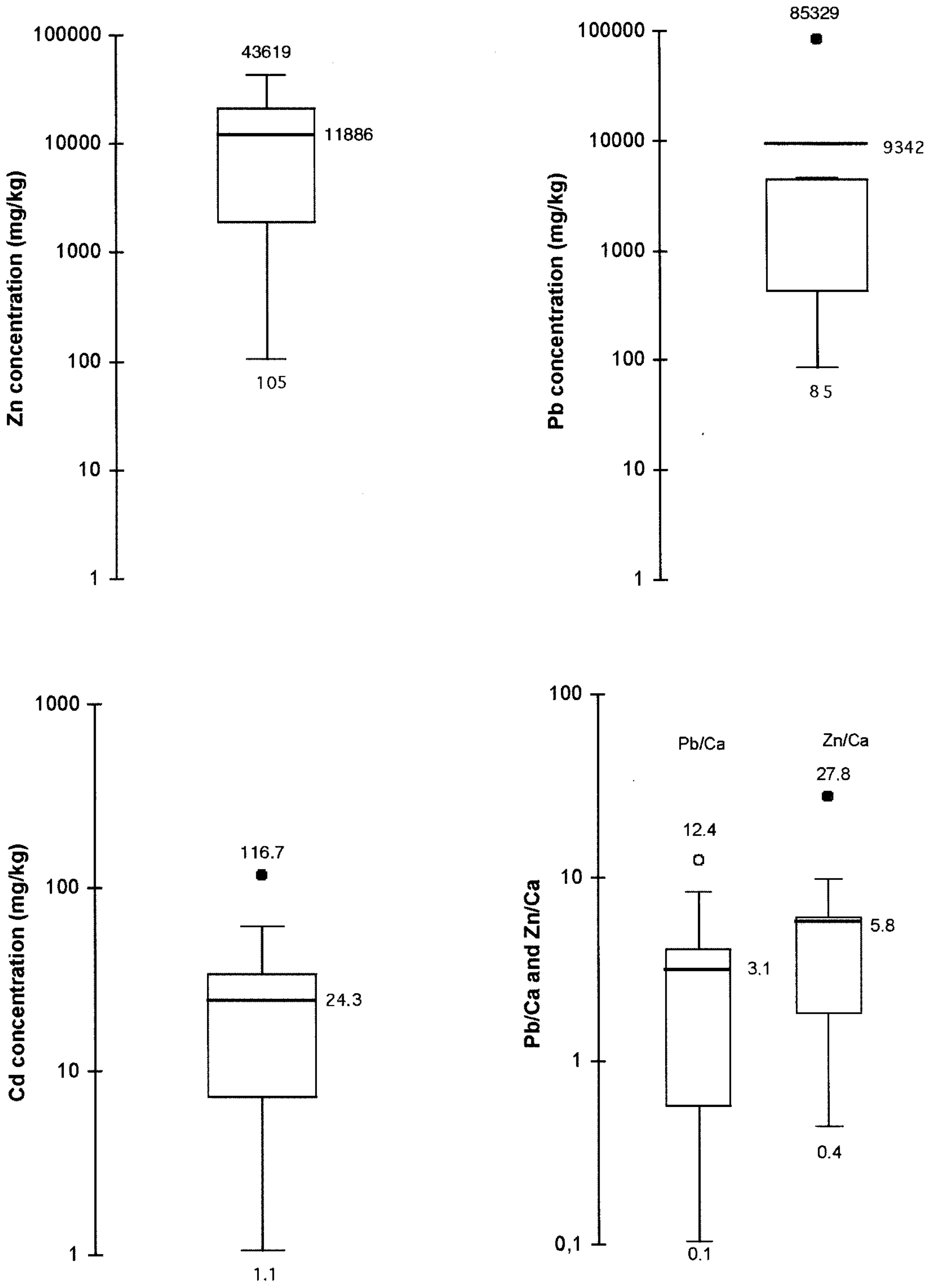

FIG. 4. — Variation in heavy metals concentration in ten populations of *V. calaminaria*. Box and wisher plots : a) Zn concentration (mg kg^{-1}), b) Pb concentration (mg kg^{-1}), c) Cd concentration (mg kg^{-1}) d) Pb/Ca and Zn/Ca ratios.

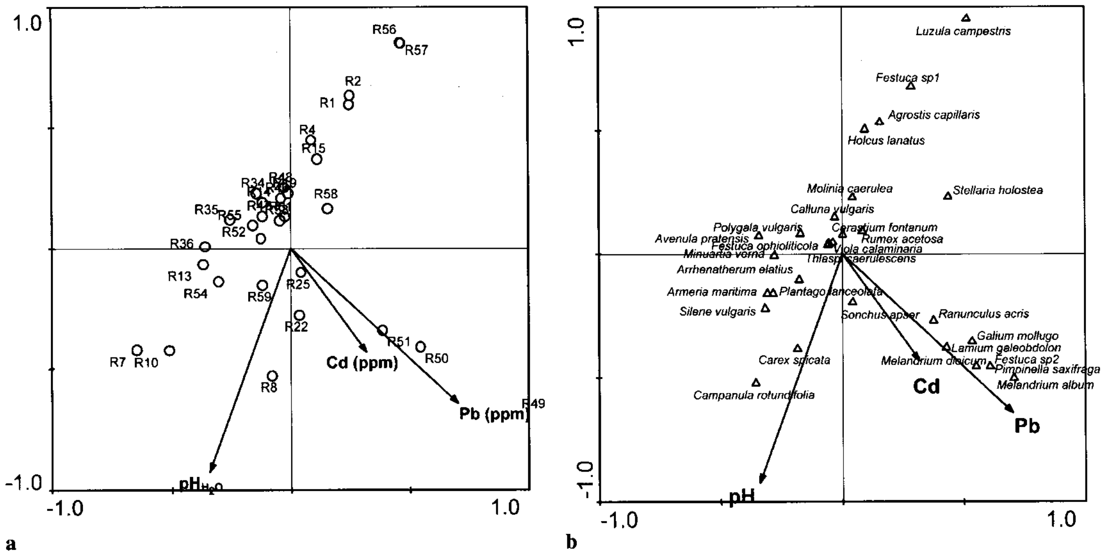

FIG. 5. — a) Quadrat-environment biplot from the CCA based on thirty quadrats from ten sites, using only significant environmental values. b) Species-environment biplot from the CCA based on thirty quadrats from ten sites, using only significant environmental values and only preferential and key species identified by TWINSPAN. Sites localisation of relevés (Rx) is given in Table 1.

Thlaspi caerulescens subsp. *calaminare* or *Festuca ophioliticola* subsp. *calaminaria*.

The same analysis was performed after removing relevés from the Huset site (R49, R50, R51), which presented a very specific floristic composition. The species-soil parameter biplots of the first two axes (Fig. 6) differed from the previous analysis. The eigenvalues were 0.328 for the first axis and 0.222 for the second axis. Together the two axes explained 17.7 % of the species composition variation and 78.6 % of the species-environment relationship. pH (highly correlated with axis 1) was the single most important variable explaining floristic variation ($P = 0.001$). Ca was the second significant variable explaining floristic variation ($P = 0.036$) evenly split between axis 1 and axis 2. Cd was only marginally significant ($P = 0.055$) and was mostly correlated with axis 2. No group structure appeared in this graph. Species tended to form a continuum along axis 1 from more acidophilous species (*Agrostis capillaris*, *Luzula campestris* or *Molinia caerulea*) to neutrophilous ones (*Campanula rotundifolia* or *Silene vulgaris*). Zn, Pb, Zn/Ca, Pb/Ca were not significantly correlated with the axes or the floristic variation.

DISCUSSION

V. calaminaria could be found in 23 metalliferous sites, which is about 55 % of all metalliferous sites present in Walloon Region. It must be noted that the entire distribution of *V. calaminaria* in Belgium was not totally covered by our study. A few populations had extended into private gardens, and could not be investigated. Also, a few isolated individuals of *V. calaminaria* are known to be present on alluvial banks of the Gueule river (GRAITSON 2004). *V. calaminaria* is also present in Germany in the vicinity of Aachen and Stolberg (SIMON 1975, 1978), in the south east of Dutch Limburg (along the Gueule river ; WILLEMS 2004). Recently, a population was also found in northern France at Auby (TONIN 2001). Mostly restricted to eastern Belgium and the Aachen region in Germany, *V. calaminaria* has always been rare. From the end of 19th century to the 1970s, new populations appeared in the vicinity of Liège as a result of increasing habitat availability caused by industrial pollution (tertiary sites ; JORTAY 1984). However, more recently, the number and size of populations have decreased because some sites have been rehabilitated.

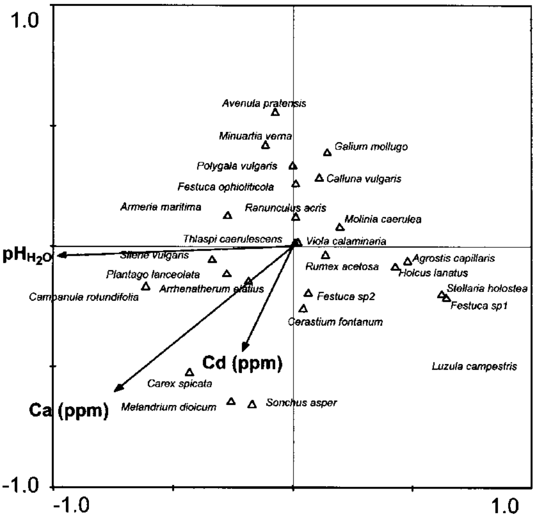

FIG. 6. — Species-environment biplot from the CCA based on twenty seven quadrats taken from nine sites (relevés of the Huset population have been removed) using only significant environmental values and only preferential and key species identified by TWINSPAN analysis.

GRAITSON (2004) found that at least six historical metalliferous sites that hosted populations of *Viola calaminaria* have completely disappeared over the last century. With the recent disappearance of four *Viola* populations found in the present study, we may thus consider that a total of ten *V. calaminaria* populations have recently disappeared. In other sites, nine populations have been partially destroyed and other populations are disturbed by grazing or mowing.

Our results confirmed that, in Walloon Region, *V. calaminaria* is endemic to metalliferous sites and is, therefore, an absolute metallophyte despite its recent geographic expansion. Soil exhibited high concentrations of heavy metals, always in excess of normal values and phytotoxicity thresholds (DE TEMMERMAN *et al.* 1984). BROWN (1994, 1995) reported that heavy metal vegetation generally develops on soil with Pb/Ca ratio greater than 1 and Zn/Ca ratio greater than 0.2. In the present study, these ratios averaged over all populations were much higher than these threshold values (mean Pb/Ca = 3.1 , mean Zn/Ca = 5.8). Interestingly, 19 samples out of 32 (60%) had values of Pb/Ca much lower or only marginally superior to 1.0 while all samples had Zn/Ca ratio higher than 0.2. This suggests that *V. calaminaria* may be restricted to soils where toxicity is mainly due to Zinc or that *V. calaminaria* may be more sensitive to Pb. In contrast, *V. calaminaria* seems less sensitive to other soil parameters. The majority of populations were found on soils with neutrophilous pH. Also, soil structure did not seem to be an important factor in the distribution of the species.

Besides the definition of the ecological amplitude, population size estimates are important information for the definition of a conservation strategy for rare species. In a first approach, larger populations may be expected to present a higher probability of long term viability due to lower genetic drift and reduced demographic stochasticity. They should then be considered as priority targets for conservation purposes. *V. calaminaria* displayed extremely variable population sizes and population structures. The largest populations were found on tertiary sites where *V. calaminaria* has experienced recent demographic expansion. For some of these sites, populations are less than 30 years old. This rather recent origin of the largest populations points to the high potential for demographic expansion of the species. Nevertheless, in the case of *V. calaminaria*, care should be taken when giving conservation priority to tertiary sites solely on the basis of population size. Interestingly, the only two relevés where *Viola* was lacking (Terbrugen) had the lowest concentrations of Zn and Cd in the soil. This may indicate that moderately contaminated sites are less suitable for *V. calaminaria*. This hypothesis will have to be verified as it might have important consequences for the definition of priorities for site conservation. Indeed, heavy metal concentrations in surface soil of tertiary sites will certainly decrease in the long term due to the current absence of atmospheric deposit. As a result, populations of *V. calaminaria* on these sites may not be viable in the long term. Threshold concentrations of Zn in the soil required for the long-term viability of *V. calaminaria* populations will have to be determined. Also, it may be hypothesised that populations from tertiary sites still reflect recent founder effect. These populations may thus present reduced genetic diversity in comparison to smaller populations in primary sites, which may have retained the initial genetic diversity of the species.

The plant communities associated to *V. calaminaria* are representative of the diversity of the metallicolous plant communities of NW Europe (Brown 2001) and of Belgium (Duvigneaud 1982, Duvigneaud *et al.* 1993) : *Violetum calaminariae typicum* and the more basicline variant of the association, *Violetum calaminariae callunetosum* ; *Agrostis capillaris* grasslands with pseudometallophytes and an impoverished set of metallophytes, a particular facies of the *Violetum calaminariae typicum* (Brown 2001) ; *Arrhenatherum* grasslands (Brown 2001). More unusual plant communities for heavy metal soils were also found. A group of relevés (from the same site) was representative of a high trophic level most probably due to leaching from adjacent cultivated fields. Another group comprised several species indicating moist soil conditions (*Deschampsia cespitosa, Phragmites australis*). This diversity of plant communities confirms the relatively large amplitude of *V. calaminaria* for soil conditions other than heavy metal concentrations. Only the *Violetum calaminariae achilletosum,* typical of more basicline soils, was not present in our relevés. Surprisingly, the CCA analysis did not indicate a strong influence of heavy metal concentrations on the floristic composition of the studied communities. In particular, Zn or the ratio Pb/Ca or Zn/Ca were not significantly discriminant variables even though they are often considered as variables that affect the flora in metalliferous sites (Brown 2001). The CCA analysis showed that pH is the main factor that affects the floristic composition of heavy metal plant communities.

From our data, it is clear that the protection of *V. calaminaria* is closely linked to the protection of calamine sites and, in particular, of sites that will retain high concentrations of heavy metals in the long term. Future work should focus on the minimal threshold of heavy metal concentrations necessary to *Viola calaminaria* establishment and development.

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