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Small scale environmental variation modulates plant defence syndromes of understorey plants in deciduous forests of Europe

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Abstract

Aim: Variation in plant defence traits has been frequently assessed along large-scale macroclimatic clines. In contrast, local-scale changes in the environment have recently been proposed to also modulate plant defence traits. Yet, the relative importance of drivers at both scales has never been tested. We aimed to quantify the relative importance of environmental drivers inherent to large and small spatial scales on the physical and chemical defence and tolerance to herbivory in understorey plant species of deciduous forests of Europe.

Location: Deciduous forests in Europe.

Time period: Present.

Major taxa studied: Forest understorey plants.

Methods: We sampled four typical ancient forest herbs (*Anemone nemorosa*, *Oxalis acetosella*, *Deschampsia cespitosa*, *Milium effusum*) along small and large spatial scale gradients (those driven by latitude, elevation, forest management and distance to the forest edge), and analysed a suite of nine constitutively expressed traits associated with overall resistance to herbivory, and their multivariate response to environmental clines.

Results: Although our study included a large gradient in macroclimate, we found variation in the local environment at small spatial scales (i.e. soil nutrient concentration and forest structural complexity) to be more important in predicting plant resistance to herbivory.

Main conclusions: In addition to macroclimatic conditions, subtle differences in forest microclimate and soil characteristics also played a major role in modulating plant

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defence phenotypes. These findings highlight the importance of the local habitat structure and environmental conditions in modulating plant resistance to herbivory.

KEYWORDS
climate, forest microclimate, forest understorey, latitudinal gradient, plant defence syndrome, plant–herbivore interactions, resource availability hypothesis

1 | INTRODUCTION

Since the origin of the first groups of phytophagous insects in the early Mesozoic (*c.* 250 Ma), plants have evolved an impressive arsenal of defence mechanisms to minimize herbivory damage (Labandeira, 1998). Although some plant species evolved complex physical structures (e.g. thorns and spines), most species are typically armed with a suite of subtle physical (e.g. trichomes) and chemical (e.g. phenolic compounds, alkaloids) defence traits that potentially increase the individuals' fitness under herbivore pressure (Agrawal, 2007; Agrawal & Fishbein, 2006; Duffey & Stout, 1996; Moles et al., 2013). Most traits, however, are not exclusively related to plant defence, but also play an adaptive role in the plant's resilience to abiotic stresses such as extreme heat events and deep shade (Moles et al., 2013). Leaf trichomes, for example, can act as a mechanical barrier for herbivores (Abdala-Roberts, Moreira, et al.,

2016; Haddad & Hicks, 2000), but also reduce leaf evapotranspiration rates in arid conditions (Gonzáles et al., 2008; Woodman & Fernandes, 1991). Such dual functional roles cause plant traits to be under multiple selective pressures from biotic (e.g. herbivory) and abiotic (e.g. climate) factors, and underpin the interactive nature of trait evolution along ecological gradients (e.g. Buckley et al., 2019; Galmán et al., 2018; Pellissier et al., 2016). This makes it extremely complex to elucidate global patterns of variation in plant resource allocation to defence against herbivores.

Nevertheless, geographic variation in plant investment in defence traits frequently follows changes in biotic and abiotic conditions (Dobzhansky, 1950; Moles, Bonser, et al., 2011; Moreira et al., 2014; Rasmann et al., 2014; Schemske et al., 2009). One of the most prominent gradients in biogeography is the increase in biotic interactions from the poles towards the equator, explained by higher temperatures, longer growing seasons and more stable

climatic conditions that facilitate speciation and population growth (Coley & Barone, 1996; De Frenne et al., 2013; Dobzhansky, 1950; Pennings et al., 2009; Schemske et al., 2009). As a result, plants native to lower latitudes usually experience a stronger predation pressure from a larger and more diverse herbivore community, which is believed to promote directional selection for increased investment in constitutive defence (Abdala-Roberts, Rassman et al., 2016; Coley & Barone, 1996; Rasmann & Agrawal, 2011; Rasmann et al., 2014; Schemske et al., 2009). However, to date, such a general pattern remains elusive (reviewed in Moles, Wallis, et al., 2011, but see Salazar & Marquis, 2012). Indeed, studies comparing inter- or intraspecific resource allocation to constitutive defence along latitudinal gradients have found variable patterns of defence investment with increasing latitude (Abdala-Roberts, Moreira, et al., 2016; Kooyers et al., 2017; Loughnan & Williams, 2019; Lynn & Fridley, 2019; Moles, Wallis, et al., 2011; Moreira et al., 2015; Moreira, Castagneyrol, et al., 2018). Furthermore, based on the assumption that the intensity of herbivory decreases with elevation, many studies predicted that investment in defence traits will decline with increasing elevation (reviewed in Moreira, Petry, et al., 2018). Some studies indeed found that plants allocate more resources to defence at lower elevations (e.g. Pellissier et al., 2016), while a significant number of studies have shown opposite trends (e.g. Rasmann et al., 2014) or no clear trends related to elevation (e.g. Buckley et al., 2019).

Such contrasting patterns in response to latitude or elevation suggest that other processes are driving variation in plant defence traits, possibly because:

1. Variation in herbivore pressure itself is independent of latitude (Moles, Bonser, et al., 2011) and elevation (Abdala-Roberts, Rassman et al., 2016; Moreira, Petry, et al., 2018), or insufficient to causally explain variation in defence traits (Buckley et al., 2019; Galmán et al., 2018; Loughnan & Williams, 2019; Moles et al., 2013; Pellissier et al., 2016; Rasmann et al., 2014).
2. Traits related to plant defence can also play a vital role in other plant functions that experience a strong selection pressure in response to environmental gradients that are unrelated – or show opposite trends – to clines in herbivore pressure across latitude or elevation (i.e. conflicting ecological drivers, Abdala-Roberts, Rassman et al., 2016; Agrawal, 2019).
3. Plant functional traits not primarily related to defence can significantly contribute to the plant defence phenotype (Agrawal & Fishbein, 2006; Carmona et al., 2011). For example, specific leaf area (SLA), foliar nitrogen (N) and phosphorus (P) concentrations are important plant functional traits for resource acquisition and nutrient cycling, but also contribute to the overall plant's attractiveness and tolerance to herbivory (i.e. the ability of plants to compensate for tissue loss), and sometimes constitute a better predictor of herbivore damage compared to classical defence traits (Agrawal & Weber, 2015; Carmona et al., 2011; Kurokawa et al., 2010; Loranger et al., 2012; Reese et al., 2016).
4. The spatial scale of sampling along latitudinal or elevational gradients (partly) mismatches the spatial scale of evolutionary processes that are postulated to drive variation in defence traits (Gaston, 2000). Forest edge-to-core transects, for example, are characterized by strong abiotic clines in temperature, humidity, light and soil nutrient availability (De Frenne et al., 2019; Meeussen et al., 2020; Murcia, 1995; Remy et al., 2016), as well as biotic clines in the floral, arthropod and gastropod community composition over less than 100 m (De Smedt et al., 2019; Govaert et al., 2020; Lantman et al., 2019).

Although all these factors determine plant resource allocation to defence or tolerance to herbivory (e.g. Coley et al., 1985; de Vries et al., 2019; Janzen, 1970; Lynn & Fridley, 2019; Moreira et al., 2016), the importance of such drivers at small spatial scales, relative to macroclimatic drivers, remains largely understudied.

Overall, there is a need for a research framework that: (a) integrates biotic and abiotic correlates of latitude and elevation (Kooyers et al., 2017), (b) accurately describes local conditions experienced by plants and herbivores at small (i.e. population level) and large (i.e. across species' distribution ranges) spatial scales, and (c) ultimately considers the multivariate nature of a plant's defence phenotype. To our knowledge, however, such a multidimensional approach has not been conducted yet.

Here we studied the relative effects of large-scale macroclimate gradients (i.e. changes in the environment across latitudes and elevations) versus small-scale forest microclimate gradients (i.e. changes in the environment along forest edge-to-core transects and due to forest management intensity). We assessed constitutively expressed physical and chemical traits associated with defence or tolerance to herbivory in four understorey plants species growing in deciduous forests of Europe. Given the regulating role of the overstorey on understorey biotic and abiotic conditions and resources, we hypothesize that small-scale changes in the environment related to forest microclimates play a major role in modulating plant defence phenotypes.

2 | MATERIAL AND METHODS

2.1 | Study species

To study spatial patterns and environmental drivers of forest understorey plant species investment in constitutive defence and tolerance to herbivores, four species (two graminoids and two dicots) were selected based on their broad latitudinal distributions (Figure 1), common occurrence in European deciduous woodlands, phenology and alternative defence strategies.

Anemone nemorosa L. (Ranunculaceae) is a spring-flowering rhizome geophyte commonly observed in ancient and undisturbed Eurasian deciduous forests and woodlands (De Frenne et al., 2011; Verheyen et al., 2003). Its latitudinal range stretches from northern Spain to northern Scandinavia (Hultén & Fries, 1986).

Oxalis acetosella L. (Oxalidaceae) is a low-growing rhizomatous perennial forest herb frequently associated with ancient deciduous
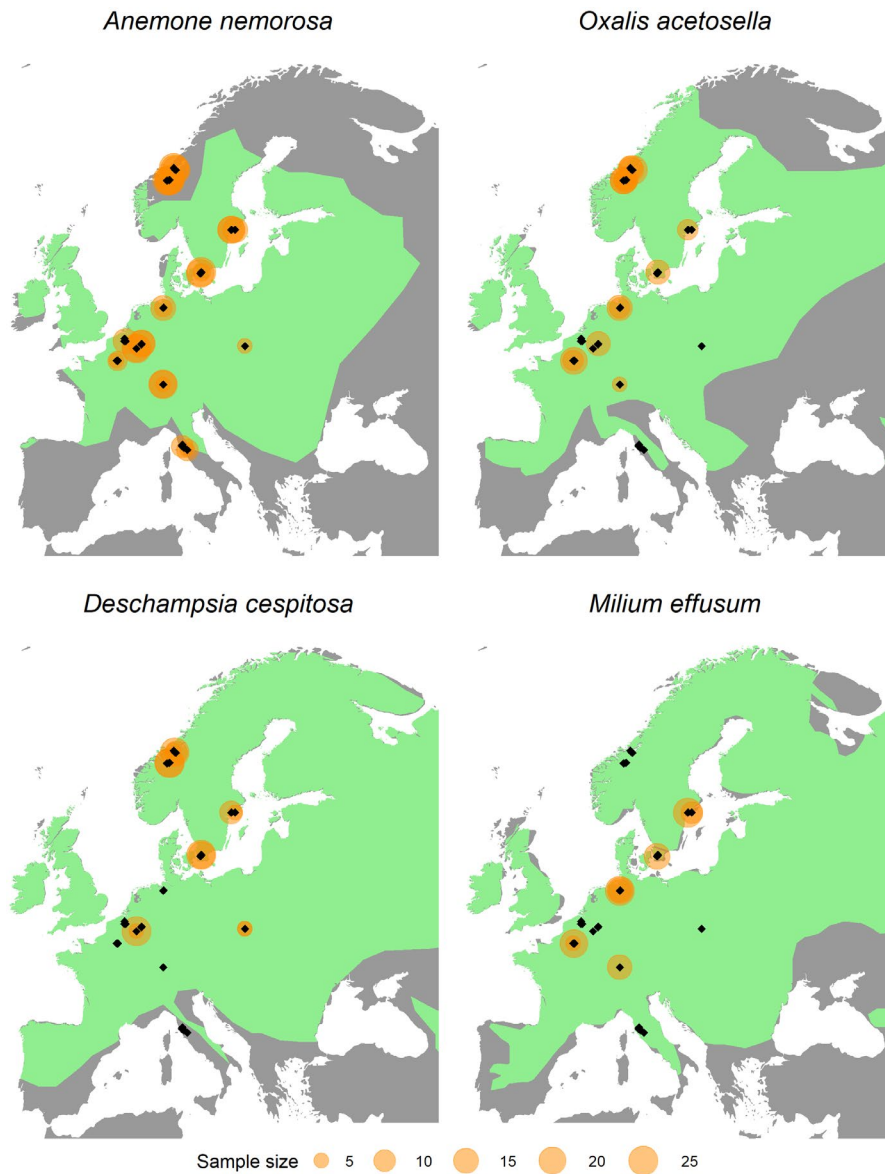
FIGURE 1 Latitudinal sample distribution relative to the species' specific distribution range (green area) for each study species (source: Hultén & Fries, 1986). Fifteen geographic sites were sampled (black diamonds). The size of the orange circles represents the sample size in each geographic site

forests. Within Europe, its distribution range stretches from central Spain to northern Scandinavia (Hultén & Fries, 1986) where it grows on drained to wet soils (Packham, 1978).

Deschampsia cespitosa (L.) P.Beauv. (Poaceae) is a common perennial grass species native to marshes, meadows and woodlands with nutrient-poor and drained to wet soils. Within Europe, its range spans from central Spain to northern Scandinavia (Hultén & Fries, 1986).

Milium effusum L. (Poaceae) is a perennial grass species associated with temperate deciduous woodlands, growing on soils with an intermediate level of soil fertility and pH (De Frenne et al., 2017). Its distribution range stretches from the Mediterranean area to northern Scandinavia (Hultén & Fries, 1986).

2.2 | Study design

We sampled 225 forest plots (3 m × 3 m) distributed over 45 south orientated forest edge-to-core transects (five plots per transect) placed along a gradient of more than 20 latitudinal degrees within Europe (latitudinal range: 42.5–63.5° N; *c.* 2,300 km; mean annual temperature range: 2.4–12.6 °C). The forest transects were distributed across nine geographic regions (central Norway, central Sweden, southern Sweden, northern Germany, southern Poland, Belgium, northern France, northern Switzerland and central Italy). Three additional elevation gradients were investigated in central Norway, Belgium and central Italy (low, medium and high, elevational range: 9–780 m a.s.l.) to capture a major part of the macroclimatic variation in European deciduous forests. As such, this study covered 15 geographic sites (nine lowland, three intermediate and three high elevation sites).

Within each of the 15 geographic sites, three forest stands with a contrasting management history were selected to represent alternative types of forest structure. The most structurally complex forest type typically had a well-developed shrub layer and a high basal area (proxy for tree stocking rate and biomass). These forests were not managed during the last 10 years and generally not thinned for > 30 years. This type of forest will be referred to as *dense forest type*.

In contrast, the most structurally simple forest type was characterized by a high canopy openness and absence of a well-developed shrub layer due to recent thinning activities (generally 1 to 4 years prior to sampling). This type of forest will be referred to as *open forest type*. Forests with an intermediate management regime and structure will be referred to as *intermediate forest type*.

All the selected forest stands covered an area larger than 4 ha and were mostly dominated by oaks (*Quercus robur, Quercus petraea, Quercus cerris*). Other common deciduous tree species such as *Fagus sylvatica, Betula* sp., *Populus* sp., *Ulmus glabra, Alnus incana* and *Carpinus betulus* were allowed as subdominant species in the canopy layer. All forest stands are ancient, that is, they were continuously forested land since at least the earliest reliable land-use maps, generally 150–300 years prior to sampling.

Forest edge-to-core gradients were studied by sampling five 3 m × 3 m plots placed along south orientated transects perpendicular to the forest edge. The forest edge was defined as the outer edge of the forest stand that meets a matrix of agricultural land, usually created by ancient deforestation. As the southern edge of the first plot on the transect was set as the edge of the forest per se, the centre of this plot was positioned at 1.5 m from the forest edge. The centres of the remaining plots were positioned at 4.5, 12.5, 36.5 and 99.5 m from the defined forest edge. As such, a total of 225 forest plots (15 geographic sites × 3 forest types × 5 plots) were sampled.

More details can be found in Govaert et al. (2020) and Meeussen et al. (2020) as we rely on the same study design.

2.3 | Plant material collection

Plant material was collected for all four study species occurring in each of the 225 plots. Sampling of plant material was conducted during early summer 2018, starting at the southernmost plots to account for differences in phenological stages across latitudes [see Govaert et al. (2020) for details].

Leaf samples and aboveground biomass were taken from five mature individuals of the four studied species (if present in the plot). Given the high degree of vegetative reproduction in many understorey plants, if possible, sampling was spaced more than 1 m apart to avoid sampling clones. All collected leaves had minor or no herbivore damage to be able to study patterns in constitutively expressed defence traits (i.e. not induced in response to herbivore attack). All leaf and biomass samples were weighed and ground after drying to constant weight at 40 °C for 48 hr. The latitudinal sample distribution ranged between 88% (*Anemone nemorosa*) and 34% (*Milium effusum*) of the latitudinal species distribution (see Figure 1 and Supporting Information Figure S2 and Table S1 for details on species-specific sample distribution across the 225 plots).

2.4 | Plant traits

Given that plants rely on multiple traits to cope with herbivory (Agrawal & Fishbein, 2006), we quantified plant defence and

tolerance in a pluralistic manner, that is, considering a suite of 10 physical and chemical traits simultaneously.

Two physical traits were assessed at the level of the individual resulting in 494 samples for *A. nemorosa*, 153 samples for *O. acetosella*, 152 samples for *D. cespitosa* and 89 samples for *M. effusum*.

Trichome density was estimated for all leaf samples of *A. nemorosa* and *O. acetosella* on the leaf surface, and for *M. effusum* on the leaf margin. *Deschampsia cespitosa* has hook-shaped trichomes positioned on longitudinal ridges at the adaxial leaf side and leaf margins. For this species, trichome density was assessed by counting the number of trichomes on one of these adaxial ridges (not on leaf margins). Counts were performed on pictures taken with a digital microscope (DinoCapture 2.0, AnMo Electronics Corporation, Taiwan). All counted densities were expressed into standard units (per cm^2 or per cm; see Supporting Information Figure S1 and Table S2 for details). Leaf trichomes can act as a mechanical barrier for herbivores (Gonzáles et al., 2008).

Specific leaf area (SLA, mm^2/mg) for each leaf was assessed by dividing leaf area (mm^2) by leaf dry weight (mg). Leaf area was measured with a LiCor LI-3000C Area Meter (LI-COR Biosciences, Lincoln, Nebraska). SLA is an important functional trait for nutrient cycling, positively related to the capacity of the plant to compensate for tissue loss (Kurokawa et al., 2010) and negatively related to the leaf physical toughness (Iddles et al., 2003; Moles, Wallis, et al., 2011), an effective defence against herbivores (Descombes et al., 2019).

Eight chemical traits were analysed based on samples that were pooled at the plot level, resulting in 103 samples for *A. nemorosa*, 33 samples for *O. acetosella*, 33 samples for *D. cespitosa* and 19 samples for *M. effusum*.

Foliar carbon (C) and *nitrogen (N)* concentrations (mg/g dry weight) were analysed for 50 mg oven-dried samples by high temperature combustion at 1,150 °C using an elemental analyzer (Vario MACRO cube, Elementar, Langenselbold, Germany). Foliar N concentrations are positively related to herbivore consumption rates (Agrawal & Weber, 2015) and provide a strong predictor for herbivore damage (Carmona et al., 2011).

Foliar C : N ratios were calculated by dividing C by N concentrations. The foliar C : N ratio is negatively related to the plant nutritional quality and generally negatively affects herbivory damage.

Foliar phosphorus (P) and *calcium (Ca)*, concentrations (mg/g dry weight) were obtained after digesting 100 mg of the sample with 0.4 mL $HClO_4$ (65%) and 2 mL HNO_3 (70%) in Teflon bombs (BOLA Digestion Vessels, Bohlender GmbH, Grünsfeld, Germany) for 4 hr at 140 °C. Phosphorus was measured colorimetrically according to the malachite green procedure (Lajtha et al., 1999). Calcium was measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS, Agilent Technologies, Santa Clara, California). When included in biominerals such as calcium oxalate crystals, this deters herbivores (Ruiz et al., 2002).

Foliar N : P ratios were subsequently calculated by dividing N by P concentrations. The foliar N : P ratio is positively related to the palatability of the plant (Kurokawa et al., 2010).

Total phenolic concentration (mg/g dry weight) was analysed as described by Moreira et al. (2014). Extractions were performed on 20 mg ground and oven-dried (45 °C) plant material in a 70% methanol solution followed by sonification for 15 min. The total phenolic concentration was subsequently analysed calorimetrically by the Folin-Ciocalteu method in a Biorad 650 microplate reader (Bio-Rad Laboratories, PA, Hercules, California) at 740 nm, using tannic acid as standard. Foliar phenolics are generally known as common herbivore feeding deterrents (Moreira et al., 2014).

Biogenic silica (BSi) concentration (mg/g dry weight) was determined following Demaster (1981): 25 mg ground and oven-dried (45 °C) plant tissue was incubated in 25 mL 0.5 M NaOH at 80 °C for 5 hr. The extracted and dissolved silica was then analysed colorimetrically on a segmented flow analyser (SAN++, Skalar, Breda, The Netherlands). High biogenic silica concentration increases leaf abrasiveness, and, therefore, deters herbivore feeding (Massey et al., 2007).

Ergot-alkaloid concentration was quantified in 13 samples of *D. cespitosa* and six samples of *M. effusum*. These samples were tested for a suite of 14 common ergot-alkaloids. The alkaloid concentration in all these samples, however, was below the detection limit and, therefore, not included in the data analyses. See Supporting Information Method S1 for more details.

These analytical measures provide a good proxy of the quantitative allocation to effective overall defence and tolerance for herbivores (Agrawal & Weber, 2015; Carmona et al., 2011; Descombes et al., 2019; Gonzáles et al., 2008; Loranger et al., 2012; Massey et al., 2007; Moreira et al., 2014). It is important to note, however, that sample sizes varied among species (Supporting Information Figure S2), which may have reduced the statistical power of the analyses for species with a lower sample size.

2.5 | Environmental predictor variables

We quantified the environment of each plot by assessing macroclimatic conditions, forest structure, understorey microclimatic conditions and soil characteristics.

Macroclimate among the 225 studied plots was quantified by high-resolution (*c.* 1 km^2) climate data extracted from the CHELSA database (version 1.2, Karger et al., 2017; Supporting Information Figure S4a,b). This database compiles high-resolution average monthly temperature and precipitation values for the period 1997–2013. We extracted the bioclimatic variables of mean annual temperature (BIO1), mean diurnal temperature range (BIO2), temperature seasonality (BIO4), maximum temperature of warmest month (BIO5), minimum temperature of the coldest month (BIO6), mean annual precipitation (BIO12), precipitation of the wettest month (BIO13), precipitation of the driest month (BIO14) and precipitation seasonality (BIO15).

Forest structure was quantitatively measured by means of single-scan position terrestrial laser scanning (TLS) using a RIEGL VZ400 (RIEGL Laser Measurement Systems GmbH, Horn, Austria) in the centre of each plot (Supporting Information Figure S6b). In detail, we used plant area index (PAI) estimates as proxies for forest structure. PAI is defined as half of the surface area of all above-ground vegetation matter per unit of horizontal ground surface area. The PAI is calculated as the integral of vertically resolved plant area per volume density (PAVD; m^2/m^3) profiles derived from TLS. As such, PAI is positively related to forest complexity and negatively related to the amount of light that reaches the forest floor. Applying TLS yields better results compared to conventional forest surveys or optical ground-based surveys, as this approach is more holistic, highly reproducible and insensitive to illumination conditions (Calders et al., 2018). Meeussen et al. (2020) provide a detailed description of the TLS methods.

Understorey microclimatic conditions can significantly differ from the overarching macroclimate and represent the conditions that are experienced by species living in the shade of trees (De Frenne et al., 2019). The magnitude of the offset between macroclimatic temperatures and temperatures below the canopy inherently couples to forest structure and distance to the forest edge. To quantify these offset values at the plot level, miniature temperature loggers (Lascar type EasyLog EL-USB-1, Lascar Electronics, Whiteparish, UK; accuracy at −35 – +80 °C: ± 0.5 °C) were installed exactly in the centre of all 225 plots at 1 m above the soil surface, covered by a radiation shield. Data loggers were installed at least 1 month prior to sampling (winter–spring 2018). Local air temperature was recorded at 1-hr intervals. For each miniature logger, we computed daily minimum (as the 5% percentile of the 24 daily measurements) and daily maximum (as the 95% percentile of the 24 daily measurements) air temperature values. Air temperatures outside the forest were recorded with the same type of miniature data loggers covered by a radiation shield and again installed at 1 m above the soil surface in each sampled geographic site (15 open sensors). All these data were used to quantify the magnitude of temperature offset during the growing season of the year of sampling (1 June 2018–30 September 2018). The offset values thus represent the magnitude of thermal buffering experienced by understorey species, and are, therefore, complementary to the average overstorey macroclimate data.

For each plot, we calculated the *mean daily minimum and maximum temperature offset values* (T$_{OFFSET}$MIN and T$_{OFFSET}$MAX) by subtracting macroclimate minimum/maximum temperatures values from microclimate minimum/maximum temperatures values (Supporting Information Figure S6a). The T$_{OFFSET}$MIN metric is positively related to the capacity of the overstorey to buffer macroclimatic temperature minima. The T$_{OFFSET}$MAX metric is negatively related to the capacity of the overstorey to buffer macroclimatic temperature maxima.

Soil pH and element concentrations were analysed for pooled samples derived from five random mineral topsoil cores per plot (0–10 cm; diameter 30 mm) (Supporting Information Figures S4c,d and S5). All soil samples were oven-dried at 40 °C for 48 hr to constant weight, ground and sieved over a 2-mm mesh. The pH-H$_2$O method was used to assess soil pH. We measured it by shaking a 1:5 ratio soil/H$_2$O mixture for 5 min at 300 rpm and using a pH meter (Orion 920A, Thermo Scientific Orion, Waltham,

Massachusetts). Soil C and N content was analysed by combusting the samples at 1,200 °C followed by measurements of the gases by a thermal conductivity detector in an elemental analyzer (Vario MACRO cube CNS, Elementar, Langenselbold, GermanyAQ24) . Bioavailable phosphorus (Olsen P; Gilbert et al., 2009) was measured by extraction in NaHCO$_3$ [according to ISO 11263:1994(E)] and colorimetric measurements were performed according to the malachite green procedure (Lajtha et al., 1999). Total Ca and Mg were measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS) after complete destruction of the soil samples with HClO$_4$ (65%), HNO$_3$ (70%) and H$_2$SO$_4$ (98%) in Teflon bombs for 4 hr at 150 °C.

Soil texture was analysed by sieving and sedimentation with a Robinson–Köhn pipette according to ISO 11277 (2009).

2.6 | Data analyses

In a first step, we explored the correlation structure among the nine herbivory resistance traits (trichome density, SLA, total phenolic concentration, BSi, Ca, C : N ratio, N : P ratio, N and P) in each of the four studied species. To do so, we performed an independent principal component analysis (PCA) for each species using the R function *PCA* from the package *FactoMineR* (Lê et al., 2008). All traits were plotted against the principal components (PC) that maximally captured the variation in the data set (i.e. PC1 and PC2). The first PC axis was subsequently set as defence phenotype axis 1 (referred to as PC1$_{DEFENCE}$). The second PC axis was set as defence phenotype axis 2 (referred to as PC2$_{DEFENCE}$). For this analysis, physical defence traits were averaged at the plot level to match the resolution of the chemical defence data. All response variables were scaled to unit variance prior to the PCA. Missing values (across all species and traits, 1.94% missing, Supporting Information Table S1) were imputed with the R function *imputePCA* from the package *missMDA* (Josse & Husson, 2016).

In a second step, PC1$_{DEFENCE}$ and PC2$_{DEFENCE}$, and the suite of nine traits were separately analysed using linear mixed-effect models (LMMs). To account for possible spatial autocorrelation (nested sampling design) in all models presented below, PC1$_{DEFENCE}$ and PC2$_{DEFENCE}$, and all chemical plant traits (BSi, Ca, total phenolic concentration, C : N ratio, N : P ratio, N and P) were modelled with the random effect term 'transect' nested within 'region' since theses samples were pooled at the plot level. All physical plant traits (trichome density and SLA) were modelled with the random effect term 'plot' nested within 'transect', nested within 'region'.

To make use of all the variation captured by the nine macroclimate and nine soil variables included in this study, we performed separate PCAs for macroclimate and soil variables. The first two macroclimate PCs explained 76% of the variation across the 225 plots (Supporting Information Figure S3a,c; Table S4a). The first macroclimate PC axis (hereafter PC$_{TEMPERATURE}$) was positively correlated to mean temperature, minimum temperature, maximum temperature and temperature range ($r > .78$). The second macroclimate PC axis (hereafter PC$_{PRECIPITATION}$) was positively correlated

to precipitation seasonality ($r = .91$) and negatively correlated to mean precipitation and minimum precipitation ($r < −.88$). The first two soil PCs explained 63% of the variation across the 225 plots of this study (Supporting Information Figure S3b,d; Table S4b). The first soil PC axis (hereafter PC$_{pH}$) was positively correlated to soil pH, soil Ca and soil Mg ($r > .78$), whereas the second soil PC axis (hereafter PC$_{NUTRIENTS}$) was positively correlated to soil C, soil N and Olsen P concentration ($r > .66$). All variables were scaled to unit variance prior to performing the PCA. The soil chemical composition variables (C, N, Olsen P, Mg, Ca) were log transformed to meet the assumptions for the PCA.

For all models presented here, continuous predictor variables, including the four new PC axes, were z-transformed to improve comparability among model parameters. In addition, for the sake of simplicity, no interactions among predictor variables were tested. Multicollinearity among predictor variables was assessed with the variance inflation factor (VIF) using the function *vif* from the R package *Car* (Fox & Weisberg, 2019).

A first set of models (design models) aimed to test the effects of the study design variables on PC1$_{DEFENCE}$ and PC2$_{DEFENCE}$, and on each of the nine plant traits in the four study species; that is, latitude, elevation, forest management type and distance to the forest edge were set as fixed effects in this group of models. Distance to the forest edge was log transformed (prior to the z-transformation) to meet model assumptions. Multicollinearity among predictor variables was weak in this model set (all VIF scores < 2.5, Quinn & Keough, 2002).

In a second set of models (environment models), the four study design variables were replaced by the environmental variables; that is, PC$_{TEMPERATURE}$, PC$_{PRECIPITATION}$, PC$_{pH}$, PC$_{NUTRIENTS}$, PAI, T$_{OFFSET}$MIN and T$_{OFFSET}$MAX were set as fixed effects in this group of models. For the set of models with *O. acetosella*, PC$_{PRECIPITATION}$ was removed from all models due to multicollinearity with PC$_{TEMPERATURE}$ (VIF » 10), for the set of models with *M. effusum*, PC$_{PRECIPITATION}$ and T$_{OFFSET}$MAX was removed from all models due to multicollinearity with PC$_{TEMPERATURE}$ and T$_{OFFSET}$MIN (VIF » 10) in the plots where these species were sampled. After model adjustment, multicollinearity among predictor variables was weak (VIF < 10), except for T$_{OFFSET}$MAX and T$_{OFFSET}$MIN in the model of *O. acetosella* with BSi as response variable (VIF < 12.5), which was tolerated.

In a final step, we performed variation partitioning analyses based on the environmental models (following Legendre & Legendre, 2012) to assess the relative importance of macroclimate (PC$_{TEMPERATURE}$, PC$_{PRECIPITATION}$), forest structure and microclimate (PAI, T$_{OFFSET}$MIN and T$_{OFFSET}$MAX) and soil variables (PC$_{pH}$, PC$_{NUTRIENTS}$) on PC1$_{DEFENCE}$ and PC2$_{DEFENCE}$, and on each of the nine plant traits in the four study species. Prior to these analyses, PAI, T$_{OFFSET}$MIN and T$_{OFFSET}$MAX were summarized by the first two PCs resulting from a PCA (capturing 87% of the total variation) to obtain three balanced groups of each two predictor variables (i.e. macroclimate, microclimate and soil). We constructed LMMs containing each time one, two or three groups of predictor variables. Model fit was evaluated with the R package *MuMIn* (Barton, 2017) by calculating the percentage of variation explained by the fixed effects (R^2_m) following Nakagawa and Schielzeth (2013). The

amount of the variation explained by the unique and shared contribution of macroclimate, microclimate and soil variables was calculated as the proportion of explained variation relative to the R^2_m of the global models (containing the three groups of predictor variables).

All analyses were performed in R (R Core Team, 2019).

3 | RESULTS

The nine traits that quantify plant resistance to herbivory were highly variable among the study species (Figure 2a, Supporting Information Table S3). *Anemone nemorosa* showed the highest trichome density, total phenolic concentration and N : P ratio. *Oxalis acetosella* showed the highest SLA. The grasses (*D. cespitosa* and *M. effusum*) had the highest concentration of BSi, in contrast with the dicots (*A. nemorosa* and *O. acetosella*), which showed the highest concentration of Ca.

3.1 | Multivariate trends in physical and chemical plant traits

The variation captured by the first two axes of the PCA, PC1$_{DEFENCE}$ and PC2$_{DEFENCE}$, ranged between 54% (*A. nemorosa*) and 70%

(*D. cespitosa*) (Figure 3). For *A. nemorosa*, *O. acetosella* and *M. effusum*, we found that defence-related traits (trichome density, BSi, Ca, total phenolic concentration and C : N ratio) were positively correlated to PC1$_{DEFENCE}$, and that traits associated with plant growth (SLA, N and P) were negatively correlated to PC1$_{DEFENCE}$. In contrast, for *D. cespitosa* we found that traits associated with plant growth (SLA, N and P) and defence (total phenolic concentration, Ca, and BSi) were all positively correlated to PC1$_{DEFENCE}$, while the plant C : N ratio was negatively correlated with PC1$_{DEFENCE}$. The PC2$_{DEFENCE}$ axis did not show clear trends related to growth and defence traits.

3.2 | Effects of latitude, elevation, forest structure and distance to the forest edge

Latitude had a positive effect on trichome density, C : N ratio and PC1$_{DEFENCE}$ in *O. acetosella*, and had a negative effect on the trichome density in *D. cespitosa* and on the P concentration in *M. effusum* (Figure 2b). In contrast, elevation was a significant predictor for a greater range of traits, including PC1$_{DEFENCE}$ in *A. nemorosa* and *D. cespitosa*. In this set of species, elevation had a similar effect on several traits: the C : N ratio increased, while the BSi and N concentration, N : P ratio and SLA decreased towards higher
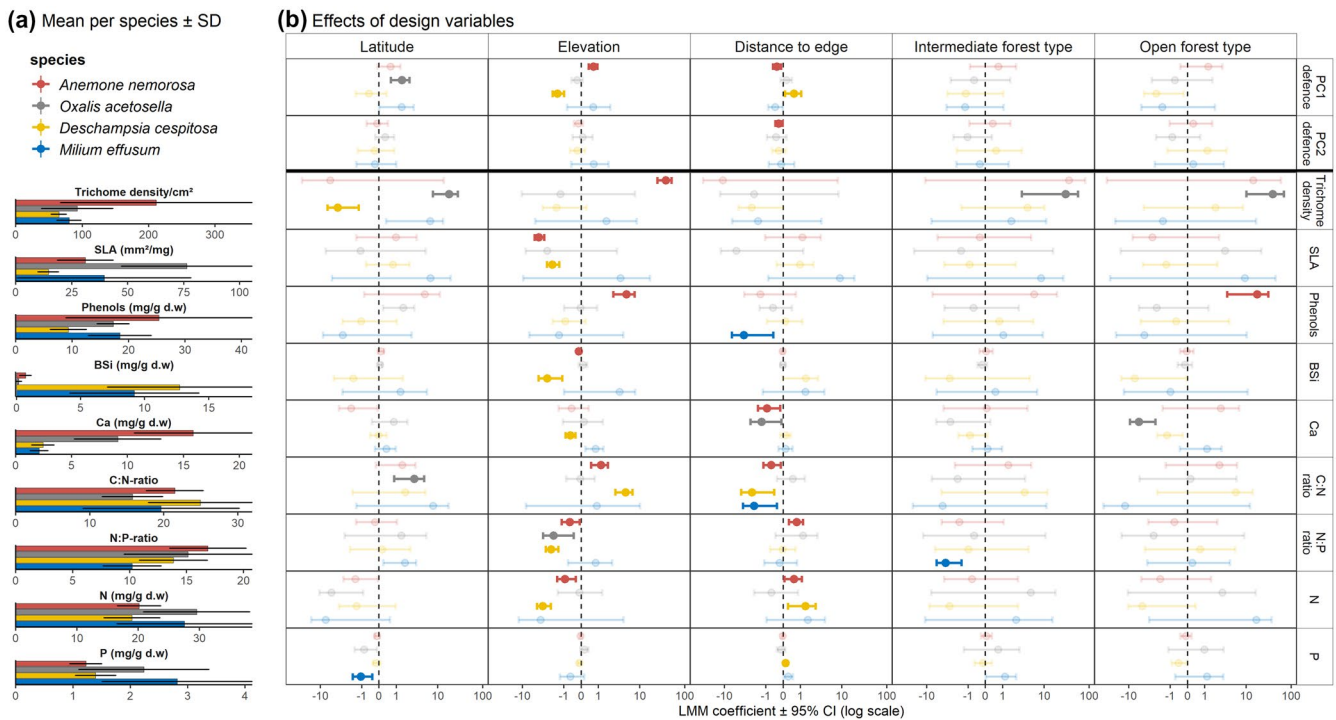

FIGURE 2 (a) Mean of plant trait values per species ± standard deviation (*SD*) across 15 geographic sites in Europe. Chemical plant traits are expressed per mass dry weight (d.w.)(b) Log transformed linear mixed effect model (LMM) coefficient estimates [± 95% confidence intervals (CI)] for latitude, elevation, distance to the forest edge and forest management type on two multivariate defence phenotypes [defence phenotype axis 1 (PC1$_{DEFENCE}$) and2(PC2$_{DEFENCE}$)], and on trichome density, specific leaf area (SLA), biogenic silica concentration (BSi), total phenolic concentration (phenols), calcium concentration (Ca), carbon : nitrogen (C : N ratio), nitrogen : phosphorus (N : P ratio), nitrogen concentration (N) and phosphorus concentration (P). The effects of 'Intermediate forest type' and 'Open forest type' are expressed relative to 'Dense forest type'. Significant effects (*p* < .05) are plotted in bold. Non-significant effects (*p* ≥ .05) are faded
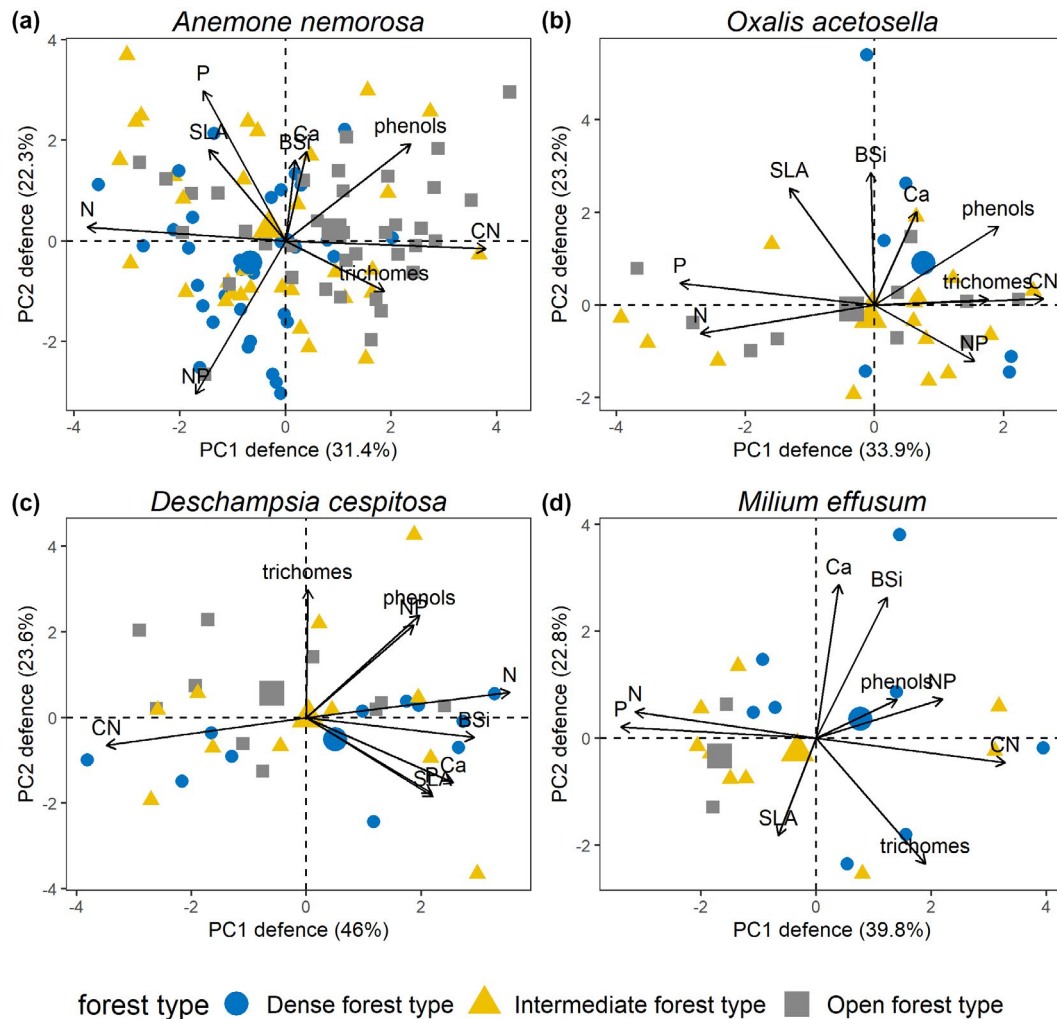
FIGURE 3 Biplots of sample coordinates and factor loading of trichome density (trichomes), specific leaf area (SLA), biogenic silica concentration (BSi), total phenolic concentration (phenols), calcium concentration (Ca), carbon : nitrogen (CN), nitrogen : phosphorus (NP), nitrogen concentration (N), phosphorus concentration (P) on the first two axes of the principal component analysis (PCA) for each individual per species. Colours and shapes refer to the type of management regime of the forest of the sample origins. The centroids of each group are represented by the larger symbols

elevations. Interestingly, we also observed significant trends in a great range of traits that could be attributed to local-scale factors: the effect of distance to the forest edge on $PC1_{DEFENCE}$ was negative for *A. nemorosa*, and positive for *D. cespitosa*. In addition, in this set of species, the C : N ratio significantly decreased with distance to the edge as a consequence of increasing N concentrations. Forest management intensity had a positive effect on trichome density in *A. nemorosa* and *O. acetosella* and on the total phenolic concentration in *A. nemorosa*, and had a negative effect on the biogenic silica concentration in *D. cespitosa* and *M. effusum*.

3.3 | Effects of environmental drivers

The two macroclimate variables, $PC_{TEMPERATURE}$ and $PC_{PRECIPITATION}$, were important predictors of several trait responses in the environmental models but we did not detect effects that were consistent

over all species (Figure 4). In contrast, $PC_{NUTRIENTS}$ had a positive effect on the foliar N concentration in all species. Although not always significant, the C : N ratio tended to decrease with increasing $PC_{NUTRIENTS}$ in *A. nemorosa* and *D. cespitosa*. Importantly, PAI was a significant predictor for the greatest range of traits: PAI had a significant effect on $PC1_{DEFENCE}$ in all species. PAI had a positive effect on the foliar N concentration in all species, and, as a consequence, negatively affected the foliar C : N ratio. We did not find consistent significant effects of $T_{OFFSET}MIN$ and $T_{OFFSET}MAX$ in the environment models.

When partitioning the proportion of the variation explained by the unique and shared contribution of macroclimate, forest microclimate and soil variables, we found that the unique effects of the microclimate variables and shared contribution of the macro- and microclimate variables accounted for the largest proportion of explained variation of $PC1_{DEFENCE}$ in most species (Figure 5). In contrast, we found that the unique effects of the macroclimate
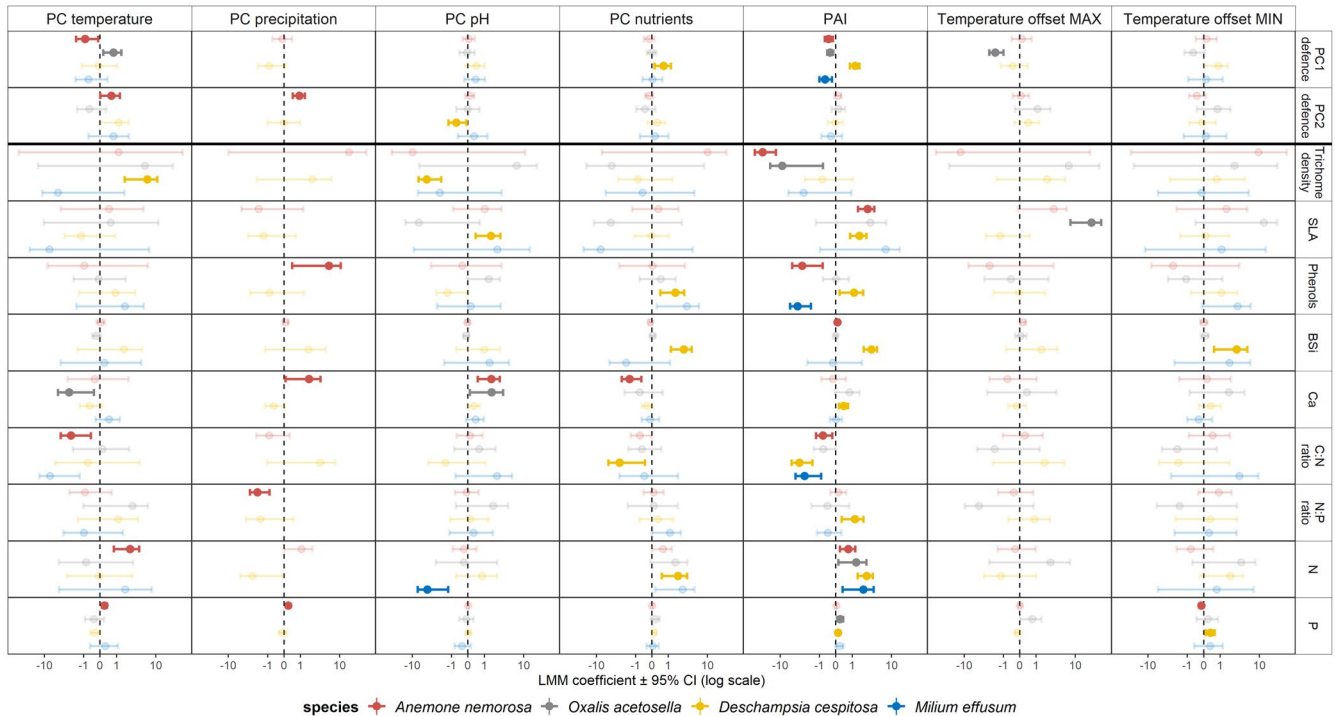
FIGURE 4 Log transformed linear mixed effect model (LMM) coefficient estimates [$\pm$ 95% confidence intervals (CI)] for $PC_{TEMPERATURE}$ (multivariate macroclimate axis 1), $PC_{PRECIPITATION}$ (multivariate macroclimate axis 2), PC_{pH} (multivariate soil axis 1), $PC_{NUTRIENTS}$ (multivariate soil axis 2), plant area index (PAI), mean daily maximum temperature offset value ($T_{OFFSET}MAX$) and mean daily minimum temperature offset value ($T_{OFFSET}MIN$) on two multivariate defence phenotypes [defence phenotype axis 1 ($PC1_{DEFENCE}$) and 2 ($PC2_{DEFENCE}$)], and on trichome density, specific leaf area (SLA), biogenic silica concentration (BSi), total phenolic concentration (phenols), calcium concentration (Ca), carbon : nitrogen (C : N ratio), nitrogen : phosphorus (N : P ratio), nitrogen concentration (N) and phosphorus concentration (P). For *Oxalis acetosella*, $PC_{PRECIPITATION}$ was removed from all models due to multicollinearity with $PC_{TEMPERATURE}$, for *Milium effusum*, $PC_{PRECIPITATION}$ and $T_{OFFSET}MAX$ were removed from all models due to multicollinearity with $PC_{TEMPERATURE}$ and $T_{OFFSET}MIN$. Significant effects ($p < .05$) are plotted in bold. Non-significant effects ($p \geq .05$) are faded

variables accounted for the largest proportion of explained variation in $PC2_{DEFENCE}$. The unique effects of the soil variables accounted for a large proportion of explained variation only for total phenolic concentration and Ca concentration in most species (Supporting Information Figure S7).

4 | DISCUSSION

This study integrates data from physical and chemical constitutively expressed plant traits related to defence and tolerance to herbivory in four common understorey plant species native to deciduous forests of Europe. We simultaneously studied the effects of the environment related to large-scale macroclimate gradients (i.e. latitude and elevation, $PC_{TEMPERATURE}$ and $PC_{PRECIPITATION}$) and to small-scale differences in forest microclimate (i.e. distance to the forest edge and forest management, PAI, $T_{OFFSET}MIN$ and $T_{OFFSET}MAX$) on a suite of nine traits associated with overall resistance to herbivory. Across all traits, we found no support for the hypothesis that plants are better adapted to cope with herbivory at lower latitudes. If anything, we found actually the opposite latitudinal pattern, with higher investment in defence traits at higher latitudes. Moreover, many of the other ecological drivers that

were investigated explained more variation in defence traits than latitude. For example, distance to the forest edge was an important predictor of the overall plant defence phenotype ($PC1_{DEFENCE}$) in most species (Figure 2b). These clines in plants' investment in defence traits were attributed to environmental gradients related to forest microclimate. We found that variation in the traits was mainly driven by variation in topsoil nutrient concentration and, more importantly, forest structural complexity (Figure 4). As such, macroclimate gradients alone are insufficient to predict plant defence phenotypes (Figure 5 and Supporting Information Figure S7). These results highlight the importance of environmental changes at small spatial scales, relative to macroecological gradients, in driving patterns of plant defence investment.

4.1 | Multiple plant traits contribute to the overall defence phenotype

Plants do not rely on a single trait to cope with herbivores (Agrawal, 2007; Duffey & Stout, 1996; Loranger et al., 2012; Moles et al., 2013). Rather, an arsenal of functional traits contribute to the overall plant defence phenotype. Most research initiatives to date, however, have focused on one or a few defence-related traits that
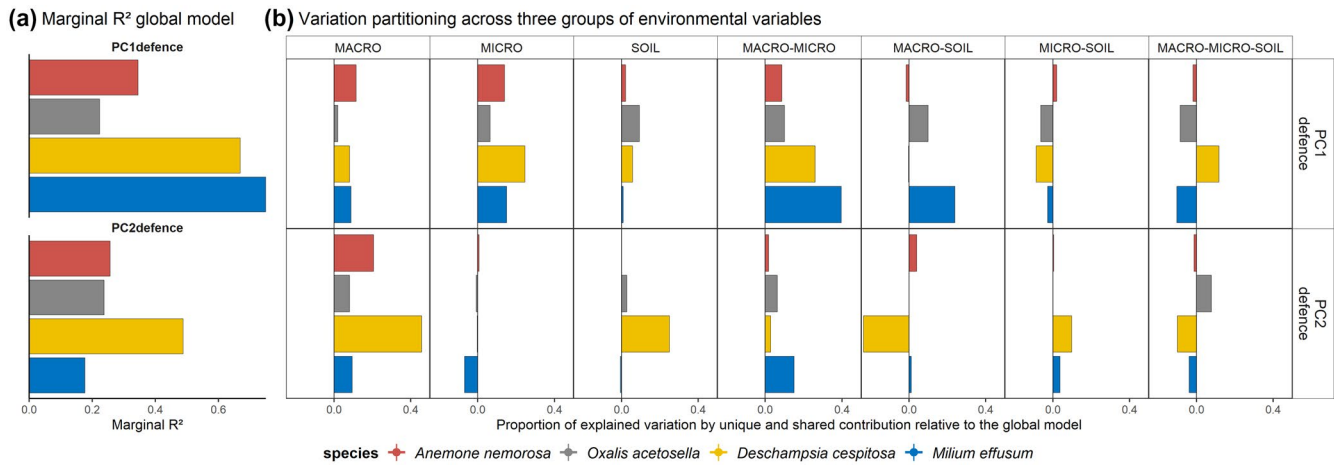
(a) Marginal R² global model **(b)** Variation partitioning across three groups of environmental variables

species ◆ *Anemone nemorosa* ◆ *Oxalis acetosella* ◆ *Deschampsia cespitosa* ◆ *Milium effusum*

FIGURE 5 (a) Proportion of the variation explained by the fixed effects (marginal R^2, R^2_m) in the global linear mixed effect models (those that include all the predictor variables) with the response variables defence phenotype axis 1 (PC1$_{DEFENCE}$) and2 (PC2$_{DEFENCE}$). (b) Results of the variation partitioning across three group of environmental predictor variables: MACRO (including the macroclimate variables PC$_{TEMPERATURE}$ [multivariate macroclimate axis 1] and PC$_{PRECIPITATION}$ [multivariate macroclimate axis 2]), MICRO (including the microclimate variables PC$_{PAI}$ [multivariate microclimate axis 1] and PC$_{T\_OFFSET\_MIN}$ [multivariate microclimate axis 2]) and SOIL (including the soil variables PC$_{pH}$ [multivariate soil axis 1] and PC$_{NUTRIENTS}$ [multivariate soil axis 2]). The amount of the variation explained by the unique and shared contribution of macroclimate, microclimate and soil variables was calculated as the proportion of explained variation relative to the R^2_m of the global models (containing the three groups of predictor variables)

are treated independently, even though appropriate multivariate applications (e.g. PCA) are available and frequently proposed as the way forward to analyse complex phenotypes (Defossez et al., 2018; Loranger et al., 2012; Moreira, Petry, et al., 2018). Here, we simultaneously analysed a suite of nine physical and chemical plant traits that contribute to herbivory resistance. For most species (i.e. *A. nemorosa*, *O. acetosella* and *M. effusum*), we found a trade-off between the group of traits associated with plant defence and the group of traits associated with plant growth (Figure 3). These results are in line with the predictions posed by the optimal defence theory (McKey, 1974). This ecological theory predicts an adaptive value of resource allocation to defence only when the associated benefits outweighs the costs of losing plant tissue. In other words, if the metabolic cost of investment in defence traits is lower than the cost of losing leaf tissue, then plants primarily invest in expensive defence traits (i.e. conservative strategy), otherwise, plants invest more in cheap and fast-growing tissue (i.e. acquisitive strategy). In this view, a species' realized niche is thus not only a product of the abiotic context of the environment (e.g. soil nutrient levels, light availability), but likely also modulated by a complex interplay among biotic (e.g. herbivores) and abiotic factors inherent to its niche.

Unexpectedly, we did not find such a trade-off for *D. cespitosa*. This species showed a positive correlation among traits associated with plant growth and total phenolic concentration, foliar BSi and Ca concentration when all the variation was summarized by PC1$_{DEFENCE}$ (Figure 3). This group of traits correlated oppositely to the C : N ratio. Trichome density was relatively unrelated to these two groups of traits. *Deschampsia cespitosa* is a species known for its extremely high levels of BSi, stored in spiny trichomes and phytoliths, which efficiently reduce herbivore damage (Davy, 1980). Due to its intrinsic

slow growth rate caused by considerable self-shading and compact growth form, this species has a poor competitive ability in forests, but is tolerant to harsh abiotic conditions (Davy, 1980). Although *D. cespitosa* tolerates a wide range of soil humidity (Davy, 1980), in natural communities, this species is completely excluded from well-drained and nutrient rich soils (Rahman & Rutter, 1980), conditions that are typically dominated by species with strongly developed acquisitive strategies. As such, we postulate that *D. cespitosa* has an intrinsic conservative growing strategy leading the ecological cost of allocating resources to defence traits to be not the major constraint for plant growth (Endara & Coley, 2011).

In summary, we conclude that the multivariate defence phenotype of plants, as defined by PC1$_{DEFENCE}$, can help to understand how clines in the environment simultaneously shape multiple plant traits associated with overall resistance to herbivory. Nevertheless, individual trait responses to environmental gradients are also important to consider to identify multiple trait functions that are potentially under selection of conflicting gradients relative to herbivory (e.g. leaf trichomes).

4.2 | Variation in plant defence and tolerance traits is associated with environmental clines at small spatial scales

Our results do not support the long-standing hypothesis that plants invest more in defence traits at lower latitudes to cope with a stronger herbivore pressure (Figure 2b). Although the latitudinal sample distribution covered 34% (*M. effusum*, 11°Δ latitude) to 88% (*A. nemorosa*, 20°Δ latitude) of the species' latitudinal ranges, these gradients are restricted

to the boreal-temperate-Mediterranean biome. Therefore, conclusions may not hold for other ecosystems such as the tropics. Nevertheless, our sampled gradient covered more than 10 °C in mean annual temperature and more than a threefold change in annual precipitation regimes. Surprisingly these factors alone played a minor role in shaping plant defence phenotypes (Figure 5 and Supporting Information Figure S7), and significant trait responses to these large-scale macroclimate gradients were often species dependent (Figure 4). Interestingly, species showed more similar trait responses to local-scale environmental gradients. Indeed, we could attribute trait variability to variation in topsoil nutrient concentration (i.e. $PC_{NUTRIENTS}$) and, more importantly, forest structural complexity (i.e. PAI). As such, not only macroclimatic conditions, but also subtle differences in forest microclimate and soil characteristics played a major role in modulating plant defence phenotypes.

Soil nutrient concentration was positively related to the foliar N concentration and negatively related to the C : N ratio. This pattern was previously described for forest understorey species growing in optimal light conditions (Elemans, 2004) and follows predictions posed by the resource availability hypothesis (Coley et al., 1985). This hypothesis states that plants living in resource-poor environments have inherently slow growth rates but highly defended tissue as the cost of replacing tissues consumed by herbivores is higher. In contrast, plants growing in resource-rich environments tend to produce cheap and fast growing tissues (Endara & Coley, 2011).

Besides soil nutrients, light is considered as the main limiting resource for understorey species in temperate forests (Landuyt et al., 2019), and directly modulates important plant functional traits such as foliar N and SLA (Blondeel et al., 2019). In our models, light availability was included as a function of forest structural complexity denoted by PAI. The amount of light that reaches the forest floor is expected to decrease with increasing PAI. We found significant positive effects of PAI on the foliar N concentration in all species. Such a modulating role of light on foliar N concentrations was previously reported for understorey species (Blondeel et al., 2019; Elemans, 2004) and could indicate a compensatory mechanism where plants allocate more N to photosynthetic tissue that is used in compounds responsible for light harvesting (Niinemets, 1997). Alternatively, foliar N concentrations can also decrease in response to increasing light availability due to an indirect effect of plant tissue nutrient dilution (Sardans et al., 2017), caused by enhanced leaf carbon uptake under elevated light conditions while nutrient uptake remains nearly constant (e.g. Blondeel et al., 2019). Indeed, we found that the C : N ratio tended to decrease with increasing PAI.

Finally, we found that PAI was an important predictor of $PC1_{DEFENCE}$ in all species. In line with de Vries et al. (2019), we conclude that the ecological costs for understorey plants to allocate resources to growth versus defence traits are predominantly determined by light availability. These results underpin the building blocks of the resource availability hypothesis (Coley et al., 1985), given that light is the main limiting resource for understorey plants (Landuyt et al., 2019).

It is important to note that our study design did not allow us to explicitly differentiate between constitutive and induced levels of trait expression as we did not quantify variation in herbivore pressure. Nevertheless, all traits are constitutively expressed and several address tolerance traits that reflect the nutritional quality of the plant. As such, biotic and abiotic factors form the entire growing season have contributed to the observed levels of these traits. Our results, therefore, apply to the overall (constitutive) plant defence and tolerance as determined by long-term patterns of herbivore pressure. Future research addressing the inducibility of both defence and tolerance traits in response to herbivore feeding will contribute to a better understanding of the importance of induced trait responses in shaping patterns along environmental gradients. Forest edge-to-core transects can in this context act as an extremely useful study system as they are characterized by strong biotic and abiotic clines over a small spatial scale (De Frenne et al., 2019; De Smedt et al., 2019; Govaert et al., 2020; Lantman et al., 2019; Meeussen et al., 2020; Murcia, 1995; Remy et al., 2016).

In conclusion, our results show clear evidence that variation in the plant defence phenotype is strongly coupled to environmental clines at small spatial scales. As such, macroclimate gradients alone are insufficient to predict plant defence phenotypes. These findings highlight the importance of the local habitat structure and environmental conditions in modulating plant resistance to herbivory, but also raise the question to what extent an individual trait response is a product of local adaptation versus phenotypic plasticity. In our study, it is likely that trait plasticity played a more important role than genetic adaptation given the short spatial scale of the forest transects (i.e. 100 m). Future research that investigates latitudinal or elevation patterns in plant defence traits should aim to quantify to what degree trait variation is linked to environmental variation at small spatial scales, and ultimately needs to include a suite of plant traits associated with overall resistance to herbivory. Such an integrated framework will contribute to our understanding of the patterns in plant defence traits along environmental gradients related to small and large spatial scales.

AUTHOR CONTRIBUTIONS

PDF, SG, PVG, CM, KV and PS designed the research. All authors collected data. PS performed the data analyses. PS wrote the manuscript, with contributions from PDF and PVG. All authors contributed substantially to revisions.

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BIOSKETCH

Pieter Sanczuk is a PhD student interested in the modulating role of forest microclimate on understorey plant responses to macroclimate warming. His research frames within the FORMICA research project (http://www.formica.ugent.be/).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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