



# Effects of soil abiotic factors and plant chemical defences on seed predation on sea fennel (*Crithmum maritimum*)

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## Abstract

**Aims** Soil abiotic factors influence plant defensive traits by shaping the costs of defence production and these bottom-up effects on plants can in turn affect insect herbivory. However, few studies have disentangled direct and indirect effects of soil abiotic factors on plant defences and insect herbivory.

**Methods** To address this gap, we tested the influence of soil abiotic factors on seed predation via changes in plant defences for sea fennel (*Crithmum*

*maritimum*), a common coastal plant in southern Spain whose seeds are consumed by specialist caterpillars (*Aethes* species). To this end, we estimated seed predation on plants across several sea fennel populations, as well as measured different types of putative chemical seed defences (phenolics, terpenes) and soil abiotic factors (macro- and micro-elements, physicochemical variables).

**Results** We found a positive association between seed chemical defences (terpenes such as  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and thymol methylether) and seed predation. In addition, a few macro- and micro-elements such as Ca, S and Sr negatively correlated with seed defences; other macro- and micro-elements or physicochemical variables had no detectable association with defences. Despite observed effects of soil abiotic factors on defences and of the latter on seed predation, there was no detectable indirect effect of soil abiotic factors on seed attack.

**Conclusions** Our findings suggest that variation in a few key soil macro- and micro-elements in coastal environments can exert an important influence on seed chemical defences in sea fennel, with potential consequences for interactions between sea fennel and seed predators.

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## Introduction

Insect herbivory is an ancient and widespread interaction (Labandeira 2007). Over evolutionary time, a striking diversity of phytophagous insects has arisen and these fall into different feeding guilds (e.g. leaf chewers and miners, cell-content feeders, piercing-sucking herbivores, root feeders, gall-makers, and seed predators; Marquis 1992), which have varying negative consequences on plant growth and reproduction (Maron 1998). Amongst these groups, pre-dispersal seed predators have particularly negative consequences for plant reproductive output and population dynamics (Kolb et al. 2007), and many of these herbivores exhibit high dietary specialization (Abdala-Roberts and Mooney 2013; Chen and Moles 2018; Gripenberg et al. 2019). Accordingly, pre-dispersal seed predators have life cycles that are highly synchronized with plant reproductive phenology (Diniz and Morais 2002; Novotny and Basset 2015) and often exhibit co-evolutionary arms races with their host plants (Berenbaum and Zangerl 1998; Thompson 2005).

Research on plant–herbivore interactions has historically focused on how the potency and diversity of defensive traits in plants has evolved in response to herbivore attack (Simms and Fritz 1990; Agrawal 2007; Futuyma and Agrawal 2009). In addition, studies have also shown that quantitative and qualitative variation in plant physical and chemical defences play an important role in determining the risk and intensity of herbivory (Feeny 1976; Marquis 1992), as well as in shaping insect herbivore communities (Richards et al. 2015; Salazar et al. 2016). Addressing these bottom-up effects of plant defences on phytophagous insects is an important task for understanding patterns of intra- and inter-specific variation in herbivory, one that also requires embracing the complex nature of plant defensive phenotypes by simultaneously studying multiple, often correlated, traits (Moreira et al. 2020a; Quijano-Medina et al. 2021).

Other well-known sources of bottom-up control in herbivory are abiotic factors, including soil macro- and micro-elements (Coley et al. 1985; Fine et al. 2004), and water or light availability (Gutbrodt et al. 2011; Abdala-Roberts et al. 2014; Ballaré 2014). Plant defence theory postulates that, since the cost of replacement of damaged tissues by herbivores is higher in resource-limited environments (e.g. low availability

of soil macro- and micro-elements, high soil salinity), plants should exhibit higher levels of defences when resources are scarce (reviewed by Stamp 2003). By increasing plant defences, soil abiotic stress can trigger host plant avoidance behaviours in herbivores or reduced feeding (Abdala-Roberts et al. 2016a; Moreira et al. 2018). To date, however, studies disentangling direct and indirect effects of soil abiotic factors on plant defences and herbivory are scarce (but see Dalling et al. 2011; Moreira et al. 2018).

Sea fennel (*Crithmum maritimum* L.) is a common halophyte herb in coastal habitats throughout Western Europe, and is attacked by seed-eating specialist caterpillars of the genus *Aethes* spp. These insects are frequently found on sea fennel plants, resulting in up to 80% of seeds attacked at some sites. In addition, the coastal sites where sea fennel grows exhibit variable but generally low availability of macro- and micro-elements, as well as high soil salinity, alkalinity, and physical stress (e.g. wind exposure). In the present study, we sampled sea fennel plants from seven populations located in the south of Iberian Peninsula, spanning a wide range of soil conditions (from sandy beaches to steep cliffs). At each population, we estimated seed predation and measured several types of seed chemical defences (phenolics, terpenes) as well as associated soil abiotic factors (macro- and micro-elements, physicochemical variables). Phenolic compounds and terpenes are putative defensive compounds that are toxic and deterrent to a broad range of phytophagous insects, and are also associated with interactions involving specialist herbivores (Mithöfer and Boland 2012). We tested whether chemical defences correlate with seed predation, if soil abiotic factors correlate with seed defensive traits, and whether any such associations between soil abiotic factors and seed defences indirectly affect seed predation. We expected that defence levels would negatively correlate with herbivory (signalling resistance against seed predation), and that plants from sites with lower resource availability and higher soil-related abiotic stress would be more chemically defended, and in turn suffer lower seed predation. By addressing multiple plant defences simultaneously, providing a comprehensive assessment of soil abiotic factors, and testing for both direct and indirect soil effects on seed predation, this study contributes to a more nuanced understanding of bottom-up effects of soil abiotic factors and plant defences on pre-dispersal seed predation.

## Material and methods

### Natural history

Sea fennel (*Crithmum maritimum* L.) is a perennial halophyte herb distributed throughout the western coasts of Europe, North Africa and the Black Sea. It flowers from June to September, and fruits mature from September to December. Plants typically bear 100–200 inflorescences at the peak of the flowering season. Each fruit contains a single dry seed from 4–10 mm long. Along its distribution, sea fennel is found in both sandy and rocky beaches, as well as cliffs, and grows frequently in sandy soils, with low availability of macro- and micro-elements and high alkalinity and salinity (Meot-Duros and Magné 2009).

Some of the most important insect herbivores on this plant are pre-dispersal seed predators of the genus *Aethes* (Lepidoptera: Tortricidae) (e.g. *A. bilbaensis*, *A. francillana*, *A. eichleri*), which specialize on several species of Apiaceae (mainly *C. maritimum* and *Carum verticillatum*). The adult female moth bores a small hole through the coat of immature fruits to lay eggs in June–July. Moth larvae then grow inside fruits feeding on the seeds and leave the mature fruit by drilling an exit hole in

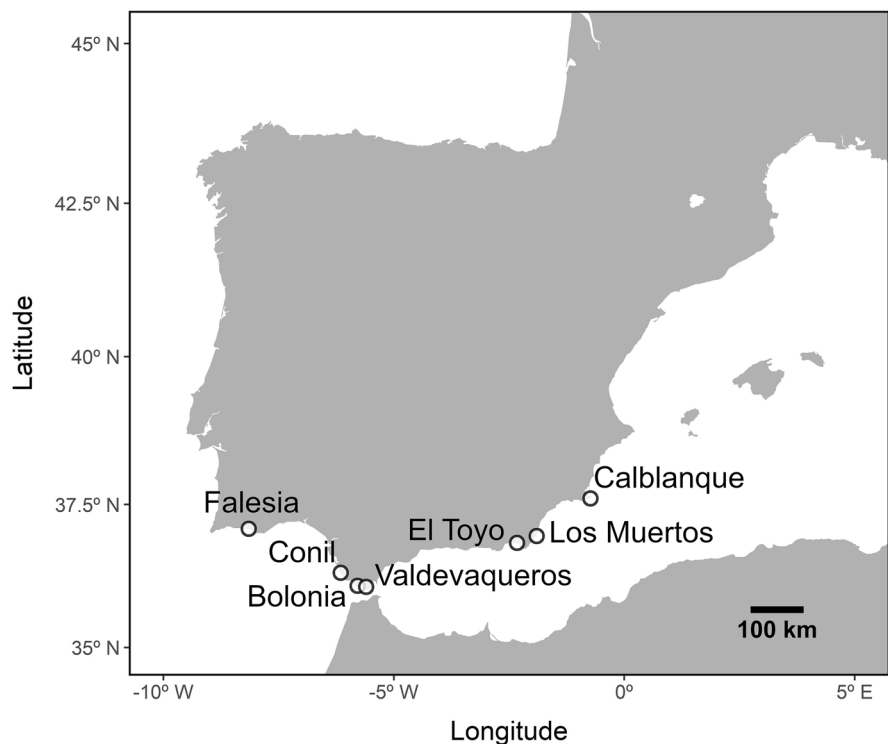
September–October. Then, larvae bore into the stems and hibernates until the following growing season.

### Field sampling

In mid-September, when plants bear both immature and mature fruits, we surveyed seven populations of sea fennel in southern Portugal and southern Spain (Fig. 1). The sites sampled had different soil characteristics and habitat topographies, including sandy or rocky beaches, and cliffs. Populations were separated by 13 to 785 km, and vary two-fold in annual precipitation and 1.2 °C in annual mean temperature. Likewise, soil abiotic factors, including macro- and micro-elements, also vary considerably across these sites (Table S1 in the Supplementary Material). Each population was comprised of at least 30 adult sea fennel plants.

At each site, we haphazardly selected 11–12 adult (reproductive) plants ( $n=83$ ) of similar height (range: 10–56 cm;  $44.59 \pm 1.62$  cm [mean  $\pm$  SE]). Plants were separated by at least 2 m, and displayed, on average,  $186.3 \pm 15.1$  (SE) umbels and  $351.6 \pm 20.6$  seeds per umbel. For each plant, we collected seven umbels with immature fruits, placed them in ice, and transported

**Fig. 1** Map showing the location of the seven populations of *Crithmum maritimum* sampled along the coasts of southern Portugal and southern Spain



them to the laboratory. Then, we opened the fruits to obtain the seed, and ground the seeds with liquid nitrogen and stored them at  $-80\text{ }^{\circ}\text{C}$  for chemical analyses. During sampling, we took special care to only collect undamaged immature fruits such that chemical defence measurements represented a rough proxy of constitutive seed defences (the influence of systemic induction if other umbels in the same plant were attacked is possible). In addition, to assess fruit predation, we collected from the same plants seven more umbels in which all or most fruits were mature and were placed in paper envelopes and transported to the laboratory where larvae continued feeding and completed their development. These samples were used to estimate seed predation (see next).

#### Estimation of seed predation

For each plant, we counted the total number of mature fruits (i.e. seeds) in each umbel and calculated the proportion of seeds attacked (number of attacked seeds / total number of mature seeds collected per plant), i.e. “seed predation” hereafter. Seed predation was overwhelmingly caused by larvae of *Aethes* species (>95% of cases; J. Cambrollé, data from this study).

#### Quantification of seed chemical defences

Phenolic compounds are feeding deterrents against insect seed predators found in many plant taxa (Dalling et al. 2020), including Apiaceae species (Berenbaum 2001). Briefly, we extracted phenolic compounds from immature seeds using 20 mg of dry material (oven-dried for 48 h at  $40\text{ }^{\circ}\text{C}$ ) with 0.25 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al. 2014). We then transferred the extracts to chromatographic vials to perform phenolic profiling. For phenolic compound identification, we used an ultra-performance liquid chromatography coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC) time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) (Bruker Compact™) (Moreira et al. 2020b). We performed chromatographic separation in a Bruker UHPLC Intensity Solo 2 C18  $2.1 \times 100\text{ mm}$   $1.7\text{ }\mu\text{m}$  pore size column using a binary gradient solvent mode consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). We used the

following gradient: 3% B (0–3 min), from 3 to 25% B (3–10 min), from 25 to 80% B (10–18 min), from 80 to 100% B (18–22 min), and held at 100% B until 24 min. The injection volume was 3  $\mu\text{L}$ , the flow rate was established at  $0.3\text{ mL min}^{-1}$  and column temperature was controlled at  $35\text{ }^{\circ}\text{C}$ . We operated MS analysis in a spectra acquisition range from 50 to 1200 m/z. We used negative (-) ESI modes under the following specific conditions: gas flow 8 l/min, nebulizer pressure 38 psi, dry gas  $7\text{ L min}^{-1}$ , and dry temperature  $220\text{ }^{\circ}\text{C}$ . We set capillary and end plate offset to 4500 and 500 V, respectively. We performed Tandem Mass Spectrometry (MS/MS) analysis based on the previously determined accurate mass and retention times and fragmented by using different collision energy ramps to cover a range from 15 to 50 eV. We recorded chromatograms at 330 nm. We identified individual compounds based on the data obtained from the standard substances or published literature including retention times,  $\lambda_{\text{max}}$ ,  $([\text{M}-\text{H}]^-)$ , and major fragment ions. We only identified phenolic compounds from two groups: flavonoids ( $N=7$ ) and hydroxycinnamic acids ( $N=3$ ). For phenolic compound quantification, we injected 10  $\mu\text{L}$  of each sample (using the same column and conditions mentioned above) in an UHPLC (Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector (Moreira et al. 2018). We quantified flavonoids as rutin equivalents and hydroxycinnamic acids as ferulic acid equivalents (Moreira et al. 2018). We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and  $5\text{ }\mu\text{g mL}^{-1}$ . We expressed phenolic compound concentrations in  $\text{mg g}^{-1}$  tissue on a dry weight basis and analysed plant-level data for total concentration of phenolics and by type of phenolic compounds (see statistical analyses ahead).

We also quantified terpenes, which are similarly considered putative chemical defences against insect seed predators in many plant taxa (Dalling et al. 2020), including Apiaceae species (Berenbaum 2001). For this, we extracted compounds from immature seeds using 300 mg of ground fresh material with 1 mL of 70% methanol in an ultrasonic bath for 20 min and stored samples at  $4\text{ }^{\circ}\text{C}$  for 24 h. We also added dodecane (Merck, #1.09658.0005) as the internal standard

solution (100 ppm of dodecane in *n*-hexane). We injected the samples (1  $\mu\text{L}$ ) onto a gas chromatograph (GC, Thermo Finnegan Trace GC Ultra, Waltham, MA, USA) with a mass spectrometer (MS) detector that was fitted with a  $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$  film thickness ZB-5MSi (Phenomenex, UK) in single ion monitoring mode (SIM:  $m/z$  68, 69, 77, 79, 92, 93, 94, 105, 119, 121, 136, 148, 161, 175) used to make visible known terpene fragments. The GC was operated in split mode ( $50 \text{ mL min}^{-1}$ ) with helium as the carrier gas (flow rate  $1 \text{ mL min}^{-1}$ ). The GC oven temperature program was: 2 min hold at  $60 \text{ }^\circ\text{C}$ ,  $10 \text{ }^\circ\text{C min}^{-1}$  ramp to  $70 \text{ }^\circ\text{C}$ , 15 min hold at  $70 \text{ }^\circ\text{C}$ ,  $5 \text{ }^\circ\text{C min}^{-1}$  ramp to  $130 \text{ }^\circ\text{C}$ ,  $30 \text{ }^\circ\text{C min}^{-1}$  ramp to  $250 \text{ }^\circ\text{C}$ , and 1 min hold at  $250 \text{ }^\circ\text{C}$ . We identified terpenes comparing their Kováts indices, calculated relative to the retention times of a series of *n*-alkanes ( $\text{C}_8\text{-C}_{20}$ , Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) analysed under the same chromatographic conditions, with those reported in the literature (Tsoukatou et al. 2001; Nabet et al. 2017). We only identified monoterpenes. For each plant, we estimated the amount of terpenes by using normalized peak areas per dry weight. The normalized peak area per dry weight of each compound was obtained by dividing their integrated peak area by the integrated peak area of the internal standard and then dividing this value by the leaf dry weight. Terpene concentration was expressed in  $\text{mg g}^{-1}$  leaf dry weight (d.w.), and we analysed plant-level data for both total concentration of terpenes and by type of terpene compounds (see statistical analyses ahead).

#### Measurement of soil abiotic factors

In mid-September, for each plant we collected top soil samples (0–30 cm depth) at a distance of 10 to 30 cm from the limit of the plant canopy projection. We measured nine soil physicochemical properties, namely: pH, water content, carbon content, electrical conductivity, and the percentage of clay, silt, gravel, and fine and gross sand. We potentiometrically determined soil pH in a 1:2.5 soil:water suspension, estimated soil water content as the proportion of mass loss after oven-drying at  $50 \text{ }^\circ\text{C}$  for 48 h, carbon content by a muffle furnace calcination ( $450 \text{ }^\circ\text{C}$  for 4 h), and electrical conductivity with a conductivity meter after mixing the soil with distilled water (1:5). For texture analysis, we removed coarse elements ( $> 2 \text{ mm}$ ) by sieving and

estimated the percentage of gravel. We then separated the soil fraction of particle size 2–0.5 mm by sieving to differentiate between gross and fine sand, and determined the proportions of fine sand, silt and clay in the  $< 0.5 \text{ mm}$  fraction by the Bouyoucos hydrometer method (Bouyoucos 1962). In addition, we measured the concentration of six soil macro-elements (Ca, K, Mg, N, P, S) and 14 micro-elements (As, B, Ba, Cr, Cu, Fe, Li, Mn, Na, Ni, Pb, Sr, V, Zn). To this end, we oven-dried soil samples at  $40 \text{ }^\circ\text{C}$  for 48 h, homogenized them by sieving to  $< 2 \text{ mm}$  in order to remove large stones and dead plant material, and ground them to  $< 1 \text{ mm}$ . We digested samples with  $\text{HNO}_3$  and HCl and analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES Varian ICP 720-ES). We estimated nitrogen concentration by the Kjeldahl method (Kjeldahl 1883). We used individual values per plant for each soil variable for statistical analyses.

#### Statistical analyses

We first assessed population variation in seed defences and seed predation using general linear models with data at the plant level (PROC GLM in SAS 9.4 System, SAS, Cary, NC) (Littell et al. 2006). Specifically, these models tested for an effect of population on seed total terpenes and phenolics, and seed predation.

We then proceeded to assess and disentangle direct and indirect effects of predictors on seed predation with a piece-wise structural equation model (SEM) using data at the plant level (Lefcheck 2016). To avoid inflating Type I error due to multiple tests, rather than individually testing for the effects of soil abiotic factors on seed defences and seed predation in the SEM, we previously summarized soil abiotic factors with a principal component analysis (PCA) using PROC FACTOR (rotation=varimax) in SAS ver. 9.4 (Moreira et al. 2015). Similarly, we also summarized seed chemical defences with PCA. The standardized  $z$ -scores from these PCs were used to test for soil abiotic factor effects on plant defences and herbivory in the SEM (see ahead). It is important to note that conventional SEM simultaneously estimates the relationships between all variables, while for piece-wise SEM the association network is broken down into different independent linear regression models

and then combined (Lefcheck 2016). This approach allows to easily incorporate specific assumptions in each of the regression models that were included in the SEM (Lefcheck 2016). The SEM allowed us to test for direct associations among soil abiotic factors and seed defences, and among seed defences and seed predation, as well as indirect associations between soil abiotic factors and seed predation through seed defences. We ran two SEMs, one for estimating direct effects and another for estimating indirect effects. For direct effects, the SEM was composed of three different linear mixed models, two modelling seed defences as a function of soil abiotic factors and one modelling seed predation as a function of both soil abiotic factors and seed defences. For indirect effects, the SEM was also composed of three different linear mixed models, two modelling seed defences as a function of soil abiotic factors and one modelling seed predation as a function of seed defences. We estimated direct associations between all variables as standardized partial regression coefficients. Indirect associations were calculated by fitting a multiple regression model between the two variables of interest (soil abiotic factors and seed predation) with any conditioning variables included as covariates (i.e. seed defences). All these models included plant population as a random factor. We assessed the significance of direct and indirect coefficients with *t*-tests. The goodness of fit of the general model was evaluated with a 'test of direct separation' based on the Fisher's C-test (Lefcheck 2016). The SEM analysis was performed in R ver. 4.0.3 (R Core Team 2020) using the *psem* function from the *piecewiseSEM* package (Lefcheck 2016).

## Results

We found significant variation among sea fennel populations for seed phenolics ( $F_{6,76}=2.78$ ,  $P=0.017$ ) and seed predation ( $F_{6,76}=5.62$ ,  $P<0.001$ ), but not for seed terpenes ( $F_{6,76}=1.80$ ,  $P=0.110$ ). Specifically, seed phenolics varied up to 1.9-fold (3.61 to 6.78 mg g<sup>-1</sup> d.w.), whereas seed predation varied up to 2.2-fold (25.81 to 56.23%) (Table 1) Terpene concentration varied up to 1.8-fold (2291.0 to 4234.8 mg g<sup>-1</sup> d.w.) (Table 1).

In the case of soil abiotic factors, the first axis of PCA explained 36% of the variation and was positively related to the concentration of Fe, Mg, Mn, Ni, and Zn (hereafter "PC1 soil") (Table 2). The second axis of PCA explained 20% of the variation in soil abiotic factors and was positively related to the concentration of Ca, S and Sr (hereafter "PC2 soil") (Table 2). The third axis of PCA explained 10% of the variation in soil abiotic factors and was positively related to water content and percentage of clay (hereafter "PC3 soil") (Table 2). In the case of seed chemical defences, the first axis of PCA (hereafter "PC1 defences") explained 32% of the variation and was positively related to the concentration of terpenes such as  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and thymol methylether (Table 3). The second axis of PCA (hereafter "PC2 defences") explained 23% of the variation in seed chemical defences and was positively related to the concentration of phenolics such as 3-caffeoyl quinic acid, 5-caffeoyl quinic acid, and ferulic acid (Table 3).

The piece-wise SEM indicated a significant negative association between PC2 soil and PC1 defences (Fig. 2), whereby plants growing in soils with higher

**Table 1** Descriptive statistics (population mean and standard error) of *Crithmum maritimum* population variation in seed terpenes (in mg g<sup>-1</sup> d.w.), seed phenolics (in mg g<sup>-1</sup> d.w.), and seed predation (in %). Data are from seven coastal populations

Population	Latitude	Longitude	Terpenes	Phenolics	Predation
Bolonia	36.088	-5.785	2291.0 ± 555.2 b	5.55 ± 0.77 ab	46.10 ± 4.35 abc
Calblanque	37.602	-0.731	2502.4 ± 555.2 ab	4.46 ± 0.77 b	42.98 ± 4.35 bc
Conil	36.314	-6.154	4234.8 ± 579.8 a	6.76 ± 0.81 a	54.62 ± 4.55 ab
El Toyo	36.836	-2.326	2744.3 ± 555.2 ab	4.52 ± 0.77 b	56.23 ± 4.35 a
Falesia	37.080	-8.148	3615.6 ± 555.2 a	6.78 ± 0.77 a	36.80 ± 4.35 cd
Los Muertos	36.956	-1.900	4029.7 ± 555.2 ab	3.62 ± 0.77 b	43.25 ± 4.35 bc
Valdevaqueros	36.067	-5.695	2808.0 ± 555.2 ab	6.64 ± 0.77 a	25.82 ± 4.35 d

sampled along southern Portugal and southern Spain. Latitude and longitude (in decimal degrees) are shown. Different letters indicate significant differences among plant populations at  $P<0.05$  based on Tukey post hoc tests

**Table 2** Results of a Principal Component Analysis summarizing the information of nine soil physicochemical properties and concentration of six soil macro-elements and 14 micro-elements. Data are from soil samples collected in the top soil (0–30 cm depth) at a distance of 10 to 30 cm from the limit of the plant canopy projection. Factor loadings, eigenvalues and % of variance explained of the three main principal components (PC1, PC2 and PC3) are shown. Values in bold show factor loadings greater than 0.80

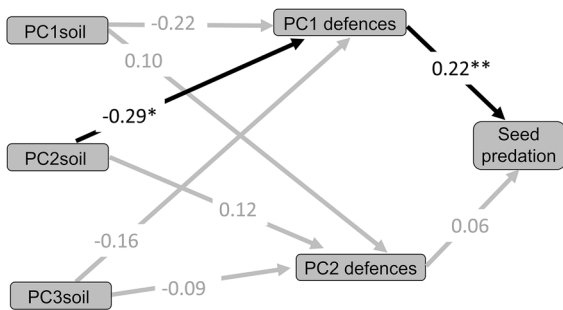
Variables	PC1	PC2	PC3
Physicochemical properties			
Water content	0.046	-0.039	<b>0.802</b>
Carbon content	0.159	0.513	0.196
pH	0.181	0.195	0.150
Conductivity	0.401	-0.387	0.240
Gross sand	0.048	0.308	-0.106
Fine sand	0.535	-0.272	0.677
Silt	0.533	-0.311	0.637
Clay	0.159	-0.225	<b>0.866</b>
Gravel	0.067	-0.513	-0.115
Macro-elements			
Ca	0.375	<b>0.859</b>	0.148
K	0.693	-0.512	0.037
Mg	<b>0.846</b>	0.369	-0.006
N	0.443	0.309	-0.010
P	0.768	0.122	-0.007
S	0.435	<b>0.820</b>	0.058
Micro-elements			
Al	0.569	-0.580	-0.173
As	0.619	0.714	-0.089
B	0.590	0.259	0.406
Ba	0.594	-0.483	-0.035
Cr	0.782	-0.309	-0.131
Cu	0.781	-0.501	-0.225
Fe	<b>0.896</b>	0.038	-0.269
Li	0.735	-0.480	-0.027
Mn	<b>0.908</b>	0.047	-0.206
Na	0.538	-0.344	-0.027
Ni	<b>0.829</b>	-0.131	-0.173
Pb	0.762	0.547	-0.122
Sr	0.308	<b>0.852</b>	0.103
V	0.752	-0.383	-0.205
Zn	<b>0.839</b>	0.445	-0.091
Eigen value	10.85	6.18	2.92
% Variance Explained	36.17	20.60	9.74

concentrations of elements such as Ca, S and Sr had lower concentrations of terpenes ( $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and

thymol methylether) in their seeds. We found no significant associations between the other two soil PCs and PC1 or PC2 defences (Fig. 2). We did, however, find a significant positive association between PC1 defences and seed predation (Fig. 2), indicating that plants with higher concentrations of some seed monoterpenes such as  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and thymol methylether were more attacked by seed predators. There was no detectable association between PC2 defences and seed predation (Fig. 2). Finally, despite the observed effect of soil abiotic factors on PC1 defences and the latter's association with seed predation, there were no detectable indirect associations between soil abiotic factors and seed predation (Fig. S1 in the Supplementary Material).

**Table 3** Results of a Principal Component Analysis summarizing the information of concentration of 11 terpenes and 10 phenolic compounds. Factor loadings, eigenvalues and % of variance explained of the two main principal components (PC1 and PC2) are shown. Values in bold show factor loadings greater than 0.80

Variables	PC1	PC2
Terpenes		
$\alpha$ -thujene	<b>0.930</b>	-0.042
$\alpha$ -pinene	<b>0.843</b>	0.136
Sabinene	0.510	0.146
$\beta$ -pinene	<b>0.876</b>	0.118
$\beta$ -myrcene	<b>0.987</b>	0.041
$\alpha$ -terpinene	<b>0.840</b>	0.077
p-cymene	0.584	-0.163
limonene	0.171	-0.075
cis-ocimene	0.541	0.179
$\gamma$ -terpinene	<b>0.936</b>	-0.058
Thymol methylether	<b>0.844</b>	-0.047
Phenolic compounds		
3-caffeoyl quinic acid	-0.220	<b>0.820</b>
5-caffeoyl quinic acid	-0.077	<b>0.897</b>
p-coumaroyl quinic acid	-0.097	0.679
Feruloyl quinic acid	-0.167	0.674
Ferulic acid	-0.107	<b>0.892</b>
3,5-di-caffeoyl quinic acid	0.190	0.625
4,5-di-caffeoyl quinic acid	0.247	0.721
Quercetin-O-hexoside	-0.060	0.717
Quercetin-7-xyloside	-0.025	-0.145
Chrysoeriol-7-O-neohesperidoside	0.057	0.207
Eigen value	6.74	4.80
% Variance Explained	32.11	22.90



**Fig. 2** Diagram showing results from a piece-wise structural equation model testing for direct associations between soil abiotic factors and seed defences, and between seed defences and seed predation on *Crithmum maritimum* individuals sampled from seven populations. Soil abiotic factors represent z-score values from a principal component analysis summarizing a suite of variables associated to soil macro- and micro-elements and soil physicochemical properties (PC1 soil, PC2 soil and PC3 soil, *see statistical analyses*). Seed defences represent z-score values from a principal component analysis summarizing a suite of compounds associated to seed terpenes and phenolics (PC1 defences and PC2 defences, *see statistical analyses*). Values next to each arrow are path coefficients (i.e. standardized partial regression coefficients). Black arrows indicate significant associations whereas grey arrows indicate non-significant associations. Explained variance: PC1 defences (marginal=0.15, conditional=0.24); PC2 defences=(marginal=0.03, conditional=0.28); seed predation=(marginal=0.18, conditional=0.59). Fisher's  $C=0.074$ ,  $P=0.964$ ,  $AICc=40.07$

## Discussion

Results from this study indicate that sea fennel exhibits a large variability in seed chemical defences, which is partially explained by heterogeneity in soil abiotic factors at the studied coastal sites. In particular, we found that the concentration of seed defences (terpenes such as  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and thymol methylether) was negatively correlated with several soil elements such as calcium, sulphur, and strontium, whereas other macro- and micro-elements or physicochemical variables had no detectable effects on seed defences. This high population variability in seed defences exerted a direct effect on seed predation, whereby we observed an unexpected positive association between seed defences (terpenes) and seed predation. Finally, despite observed soil effects on seed defences and of the latter on seed predation, there was no detectable indirect effect of soil abiotic factors on seed predation. Overall, these findings suggest that

variation in a few key soil macro- and micro-elements (and the biotic or abiotic processes underlying their variation) can exert an important influence on sea fennel chemical defences, with potential consequences for sea fennel-seed predator interactions.

There was a negative association between several soil macro- and micro-elements such as calcium, sulphur, and strontium and the concentration of seed defences (terpenes such as  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -myrcene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, and thymol methylether), suggesting that sea fennel plants growing in soils with lower availability of these elements were more highly defended. Calcium (Ca) is known to be a critical macro-element for the formation and development of new plant tissues (reviewed by White and Broadley 2003) whereas sulphur (S) is essential for the formation of chlorophyll (reviewed by Zhao et al. 2008), and both elements are abundant in coastal soils (Phleger 1970; Howarth 1984). In the case of strontium (Sr), this micro-element is found abundantly in soils in the form of sulphate and carbonate minerals, and the uptake by roots appears to be related to mechanisms of mass-flow and exchange diffusion (Sasmaza et al. 2020). It also displays complex interactions with calcium, but usually cannot replace Ca in biochemical functions (Walsh 1945). Plant defence theory holds that plants adapted to nutrient-poor environments invest more resources in defences as the cost of replacement of herbivore-damaged tissues is higher under stressful conditions (reviewed by Stamp 2003), which could explain the negative association between seed defences and these soil variables. In addition, it is also possible that sea fennel plants growing at sites with lower amounts of these macro- and micro-elements allocate less to growth and in turn exhibit higher terpene-based defences via growth-defence trade-offs (Fine et al. 2006; Sampedro et al. 2011). Unfortunately, we currently cannot differentiate these possibilities because this species is perennial and in situ measurements of plant size at hand (e.g. height, volume) cannot separate between effects of resources on growth and plant age. Follow-up work in situ or ex situ (e.g. greenhouse, common gardens) with planted individuals of known age for which we manipulate soil concentrations of these macro- and micro-elements and measure defences are needed to understand the relationships between resources, plant growth, and defences for sea fennel.



The positive association between seed predation and seed defences (terpenes) mirrors findings from previous work on insect herbivory and plant chemical defences, particularly for specialist herbivores (e.g. Castillo et al. 2014; Abdala-Roberts et al. 2016a; Ochoa-López et al. 2020). One possible explanation is that terpenes (including volatile compounds) serve as host finding cues by *Aethes* ovipositing females, as shown for other seed-eating species of Tortricidae for which specific monoterpenes have been shown to attract ovipositing females (e.g. Wearing and Hutchins 1973; Sutherland et al. 1977). Likewise, similar findings have been reported for other groups of specialist insect herbivores such as Coleoptera (e.g. bark beetles, Erbilgin and Raffa 2000; Seybold et al. 2006). In addition, *Aethes* caterpillars may obtain physiological or immunological benefits from feeding on host plant toxins (reviewed by Erb and Robert 2016), including terpenes (e.g. Becerra 1997) as well as other types of compounds such furanocoumarins which are also common in the case of Apiaceae (e.g. Carroll et al. 1997; Carroll and Berenbaum 2006). These benefits include boosted immunological responses against pathogens (e.g. Singer et al. 2014; Barthel et al. 2016; Garvey et al. 2021) as well as defence against parasitoids and predators via metabolite sequestration (Katsanis et al. 2016; Kelly and Bowers 2018; Ochoa-López et al. 2020). It should be noted, however, that although we used only undamaged seeds for chemical analyses, systemic induction due to attack on other umbels of the same plant could have resulted in higher chemical defences than expected in undamaged seeds (Abdala-Roberts et al. 2016b; Moreira et al. 2018), potentially leading to a positive correlation between seed predation and terpenes. Past studies have found terpenes to be induced upon feeding by other Tortricidae species (e.g. Hern and Dorn 2002; Giacomuzzi et al. 2016) and spatial variation in herbivore pressure could be an important driver of population variation in sea fennel defences as shown in other plant taxa in Apiaceae (Berenbaum and Zangerl 1998) as well as species in other families (Züst et al. 2012; Abdala-Roberts et al. 2016b). Unfortunately, the methodology used in the present study does not allow us to differentiate these two explanations and unambiguously assess bottom-up effects of plant defences. Further work involving sampling designs in which the timing of seed collection is conducted prior to the onset of seed predation to gain insight into how pre-existing (constitutive) defence levels affect seed predation. At the same time, experimental manipulation of herbivory in situ or under greenhouse

conditions, coupled with caterpillar bioassays (including sequestration of secondary metabolites), are needed to understand the consequences of plant induced responses for this interaction.

While phenolics and terpenes are commonly studied as defences in the context of plant–herbivore interactions, they may also serve for other functions, particularly in the context of abiotic or physical stress. For example, phenolics are expressed in higher levels under conditions of high light availability to protect leaves from damaging effects of excess light (Abdala-Roberts et al. 2014; reviewed by Ballaré 2014). Likewise, terpenes have also been shown to be involved in plant protection against extreme abiotic conditions such as drought or high temperatures (e.g. Llusà and Peñuelas 1998), indicating also a role in abiotic tolerance and stress responses. In addition, recent work shows that salt-stressed plants can prime physiological responses to better cope with salinity stress in neighbouring plants via airborne terpenes (Caparrotta et al. 2018). In this sense, manipulations of abiotic factors such as salinity, temperature, or soil humidity could be coupled with manipulations of soil nutrients and seed performance (viability, germination) to understand the relationship between different components of the abiotic environment, seed (and other tissue) chemical traits, and plant fitness.

Despite soil macro- and micro-elements negatively correlated with seed defences (terpenes) and these metabolites positively correlated with seed predation, there was no detectable indirect association between soil abiotic factors and seed predation. Recent work of ours investigating indirect effects of soil and climatic factors on herbivory via plant defences in other plant taxa has shown mixed evidence. For example, in agreement with present findings, precipitation negatively affected leaf pubescence and positively affected leaf-chewer herbivory in wild cotton, but there was no indirect effect of precipitation on herbivory (Abdala-Roberts et al. 2019). However, for English oak (*Quercus robur*) we found that temperature and precipitation were negatively associated with leaf and seed defences (respectively) and this led to positive indirect effect on seed predation (Moreira et al. 2020b). Likewise soil physical properties (e.g. porosity) positively correlated with oak chemical defences and this led to an indirect negative effect on leaf herbivory (Moreira et al. 2018). Results thus far appear to be system-specific depending on the plant taxa

studied or type of defences or abiotic factor looked at, thus warranting more studies before general rules for the strength of indirect abiotic effects on herbivory can be inferred. Identifying the most important abiotic factors affecting plant defences in a given system (e.g. limiting macro- and micro-elements or soil physical properties), as well as key physical or chemical defences with known impacts on associated phytophagous insects (e.g. specialized chemical defences against focal insect herbivores or broad-spectrum metabolites against diverse generalist insects) are needed in order to achieve more robust tests of indirect effects of abiotic forcing on herbivory.

We envision a couple of avenues for future research on abiotic bottom-up effects on herbivory in sea fennel as well as other plant taxa. First, expanding research to include a greater number of populations replicated across different coastal habitats in order to assess spatially varying abiotic factors driving variation in herbivory. Accounting for other abiotic factors such as climatic or mechanical damage due to wind is also warranted to achieve a more comprehensive evaluation of abiotic sources of spatial variation in herbivory. Second, conducting within- and among-population manipulative assessments of macro- and micro-element effects (mimicking observed variation in situ) on chemical defences, both constitutive and induced, under controlled and/or in situ conditions. These tests would also involve measuring effects on herbivory, namely identifying key metabolites (volatile or non-volatile) influencing seed predator host plant preference and seed consumption, as well as testing for indirect effects of abiotic manipulations on these herbivore responses.

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**Author contributions** Formulated the idea of the manuscript: XM, JC, LAR. Designed the experiment: JC, IMPR, LM. Performed the field sampling: JC, IMPR, LM. Performed plant and soil measurements: XM, JC, IMPR, LM, RMN, AGG, MF. Contributed reagents/tools: XM, JC. Analysed the data: XM, CVG. Wrote the first draft of the manuscript: XM. Contributed critically to the writing: LAR, JC, IMPR, LM.

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## Declarations

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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