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Yearly variation in allelopathic compound production along a climatic gradient

A case of study of Empetrum nigrum

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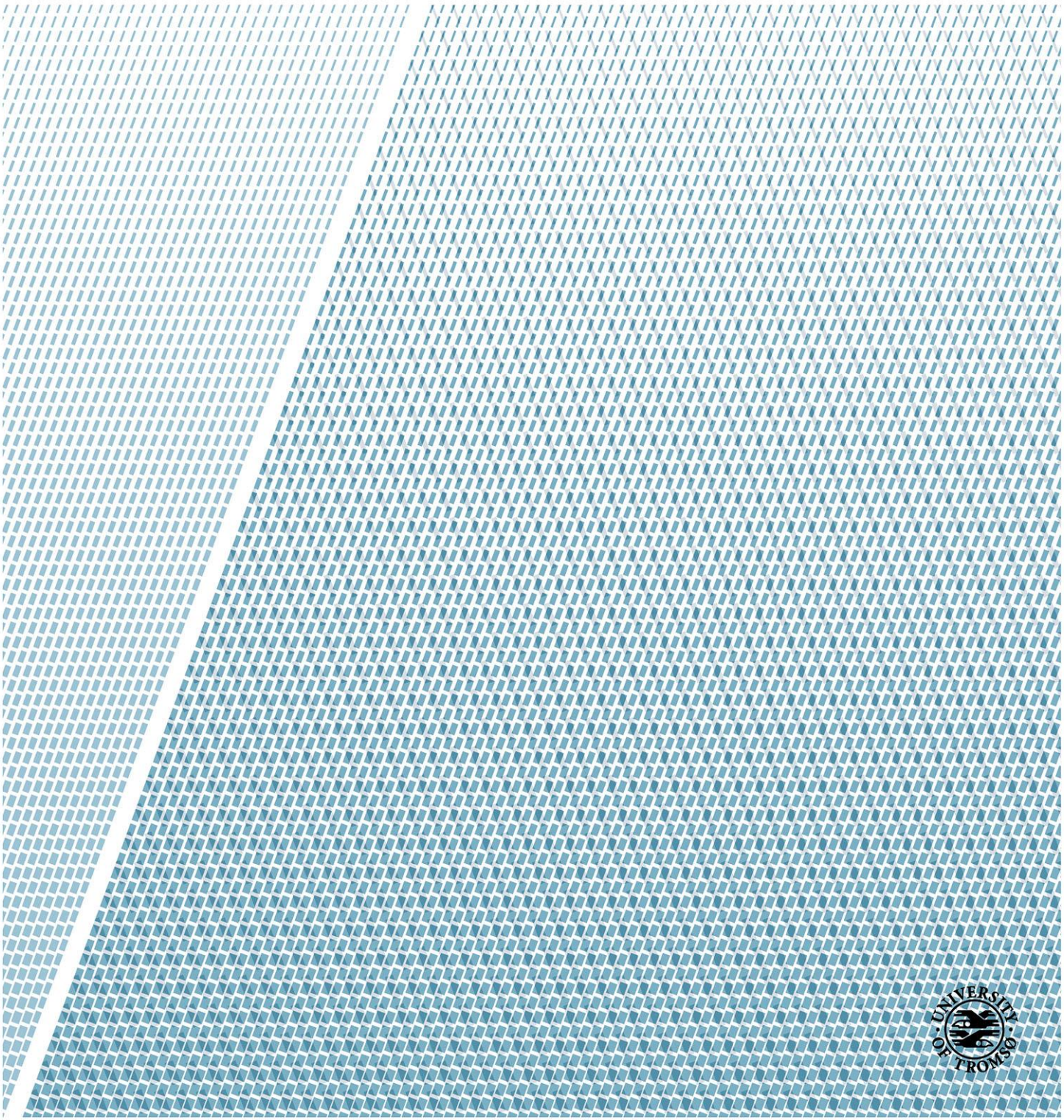

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Tromsø, (it was about time to finish) November 2017

Mikel Moriana Armendariz

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Abstract

Empetrum nigrum is a plant common in northern ecosystems with capacity to produce allelopathic compounds, which among other effects inhibit seed establishment and germination of other plants. Some of the most studied compounds regarding this effect are batatasin-III and phenolic acids, among them caffeic acid, which account for a large proportion of the leaf's biomass. 5 random sites were established following a climatic gradient, and first year shoots were collected during 7 years. The plant's antioxidant effect was studied as proxy of allelopathy, and shoot length was measured for the possible trade-offs between production of secondary metabolites and growth. The effect of climatic variables on the production of batatasin-III and caffeic acid was assessed, and the plant's antioxidant activity and shoot growth were studied in accordance to the metabolite production and the weather conditions. Plants had higher concentration of batatasin-III in sites with low temperature, high number of freezing days during winter or both. Nevertheless, a positive relation between temperature and production of batatasin-III was found at the site level. Caffeic acid and antioxidant activity were positively related, however neither the weather variables studied explained their pattern. Shoot length was related to the year air temperature, but not to the production of batatasin-III or caffeic acid. In conclusion, this study shows that an increase of yearly temperature is likely to lead to an increase in batatasin-III production at the site level, but that no effect would happen to the growth of first year shoots. We were not able to find any impact of weather conditions on concentration of caffeic acid and on antioxidant activity.

Keywords: *Empetrum nigrum*, batatasin-III, allelopathy, caffeic acid, antioxidant activity, plant growth, weather effects.

Introduction

Secondary metabolites have evolved as a part of the organism's survival strategy (Williams *et al*. 1989). The optimal defence theory proposes that the defence of different plant tissues depends on their value to the plant, the costs of defence and the risk of herbivore or pathogen attack (Rhoades and Cates 1976). Metabolites are expressed as a result of stimuli, and target specific receptors (Christophersen 1991). For that reason, the more metabolites a plant possesses, the more receptors it is capable of targeting. This idea is expressed by both the lottery principle and the screening hypothesis. The lottery principle (Williams 1975) suggest that a more diverse collection of secondary metabolites increases the probability of a plant's phenotype matching it environment; while the screening hypothesis (Firn and Jones 1996) adds that this advantage is more important in inherently variable environments, across both spatial and temporal scales.

However, maintaining a high diversity of metabolites imposes strong energy costs, which can reduce the plant's ability to survive (Baldwin 1998). In that way, strong directional selection can lead to an accumulation of active metabolites at the expense of inactive ones (Moore *et al*. 2014). When ecological circumstances are favourable the synthesis of plant secondary metabolites can be completely switched off, to be resumed when required (Moore *et al.* 2014). Scarcity of soil resources, increased competition or increased herbivore activity lead to the production of secondary metabolites as means of defence, attack or make nutrients available (Mitra and Baldwin 2008, Inderjit *et al.* 2011).

Plants are the main primary producers in an ecosystem, and therefore have the ability of up-regulate the system (Oksanen 1990), from controlling herbivore feeding activity (Coley *et al.* 1985), to determining soil flora and decomposition patterns (Flanagan and Van Cleve 1983; McClaugherty 1983). One way plants do this is by means of allelopathy, the interference mechanism by which live or dead plant material, including litter, releases chemicals which exert an effect (usually negative) on other plants (Rice 1984). Allelochemicals also have effects on other levels of the ecosystem, as they make plant tissues (both live and dead – litter) less palatable and, therefore, less desirable by herbivores and soil organisms (Hobbie 1992). This dual ability to resist herbivory and produce low quality litter is expected to have strong ecosystem effects (Pastor *et al.* 1988). Plants rarely rely on only one compound for their defence, but they produce an amalgam of molecules, which results in a mixture of synergistic and antagonistic effects (Hadacek 2002). Plant populations usually vary in presence-absence of the metabolites and, more commonly, in quantitative variation of the metabolite concentrations, which results in variation in richness and α-chemodiversity (Moore *et al*. 2014). Two strategies can be found: production of a less variable metabolite spectrum, with certain compounds with a specific function in high concentrations; or production of a more diverse metabolite spectrum, with no compounds standing out (Simons 2009). Plants following the first pattern may have a high arithmetic fitness across most years, but failing when facing unexpected factors or under heavy pressure. Plants following the second pattern, meanwhile, may have less fitness most of the years, but will survive under unexpected circumstances and might have a higher survival in the long term (Moore *et al.* 2014).

Many studies assessing allelopathic effect are focused on the direct effect a metabolite has on the development of another plant (e.g., seed establishment and germination, root and shoot growth. Nilsson and Zakrisson 1992, González *et al.* 2015). However, once a chemical compound reaches the soil, interactions with the environmental conditions, other chemicals, and the biota in itself are decisive for the allelochemicals' performance (Inderjit and Weiner 2001). Furthermore, ecological activity can vary with the activity of phenolics and oxidants, even when the concentration and composition of phenolics are constant. The particular mode of action will

depend on the redox state of the phenolics, which varies according to the pH of the soil. (Appel 1993). Oxidized phenolics may oxidize other phenolics of lower redox potentials, initiating chain reactions of oxidation and polymerization (Dennisov and Khudyakov 1987). Therefore, effects of metabolites on the redox state of the soil, by antioxidant/oxidant activity or by quenching other chemicals and inhibiting their functions, are important to study.

Climate is an important factor affecting many aspects of a plant during its life cycle, and thus it is likely to affect the plant's metabolic production. Higher temperatures lead to a longer growing season and can reduce some of the stress plants have to face in northern ecosystems. Longer growing season and reduced stress could result in additional resources to be invested either in growth or in secondary metabolites (Herms and Mattson 1992). A recent study in *Quercus robur*, also found secondary metabolites, in this case plant defences, to be affected by climate, with plants in more northern latitudes showing a bigger investment in defences (Moreira *et al.* 2017).

The ericaceous *Empetrum nigrum* is a species with a quasi-circumpolar distribution (Walker *et al.* 2005) and it is widely distributed in Northern Scandinavia, where it grows in dense mats of nearly monospecific vegetation (Bell and Tallis 1973). These mats allow very few other species to grow; thus, once established, and even though its growth rate is very limited, *E. nigrum* will creep its way into dominance (Tybirk *et al*. 2000). It has been shown to have effects at different ecosystem levels. A study by Bråthen *et al*. (2010) showed how *Avenella* and *Solidago* struggled to grow under nutrient stressed conditions, which were present in humus collected from sites completely dominated by *Empetrum*. This is likely due to the phytotoxic compounds produce by *Empetrum*, which can also effect herbivory, as these compounds are poor in nutrients and make the leaves highly unpalatable. This can affect, in turn, habitat use as reindeer is a selective species looking for sites with the best forage quality (Iversen *et al.* 2014).

An important feature of *Empetrum nigrum* are its leaves. *E. nigrum* belongs in the *Empetraceae* family, in the order Ericales, and, as such, presents the coriaceous and convoluted leaves typical of this group (Anderberg 1994). The leaf is folded in such extent that it creates a cavity in its abaxial side. This cavity is saturated with water vapour, and provides good conditions for microorganism growth. In order to protect itself, *E. nigrum* possesses glandular trichomes within this cavity that produce and release toxic metabolites, of phenolic nature in many cases (Muravnik and Shavarda 2012). These compounds can leak into the soil by means of rain, snowmelt or litter deposition, and direct their toxic effect towards other organisms (e.g. soil microflora, neighbouring plants) (Zackrisson and Nilsson 1992, Nilsson *et al.* 1998).

Nilsson *et al.* (2000) found differences in the metabolic production of *Empetrum nigrum*. While the subspecies *hermaphroditum* produced batatasin-III, the subspecies *nigrum* produced a molecule similar in structure but with reduced allelopathic effect. These subspecies have a

different distribution (*hermaphroditum* in Northern Europe, *nigrum* in Central Europe. Anderberg 1994), which suggests metabolite production might be affected by the environmental setting. The aforementioned batatasin-III is one of the most studied secondary metabolites regarding *Empetrum nigrum*'s allelopathic effect, and it has been discovered to account for a considerable part of the total allelopathic effect of the species (e.g., 28% in a study by Odén *et al.* 1992). It has been shown to inhibit seed germination and establishment of several species (González *et al.* 2015), as well as to have some general phytotoxic effects (Nilsson *et al.* 1998).

Empetrum nigrum has been shown to produce an array of different secondary metabolites (Muravnik *et al*. 2012). Phenolic acids are an important resource for *E. nigrum* (Nilsson and Zackrisson 1992, Wardle *et al.* 1997), though their relation with phytotoxicity is less pronounced (Nilsson *et al.* 1998). One of the most abundant phenolics in young leaves of *Empetrum* is caffeic acid, accounting for as much as 50% of the total phenolic load (Gallet *et al.* 1999). Caffeic acid has been shown to be part of the inhibitory effect exerted by other species, like *Patrhenium hysterophorus* (Kanchan and Jayadrancha 1980) and *Euphorbia esula* (Barkosky *et al*. 2000), as well as to inhibit phosphate and potassium uptake (Glass 1973, Glass 1974). Other common metabolites are chalcone derivatives, with two of them, called empetroxepin A and B, having been shown to exert antimycobacterial activity (Li *et al.* 2015)

Here I hypothesize that production of secondary metabolites (batatasin-III and caffeic acid) by *Empetrum nigrum* varies according to climatic conditions, and for testing this 5 sites were randomly selected following a climatic gradient from ocean to inland. Within each site 3 plots were randomly chosen and sampled during 7 years. I hypothesize that allelopathic effect, measured as the plant's antioxidant activity, and productivity, measured as shoot length also vary between sites, as both the biotic and abiotic conditions are likely to differ. *Empetrum* has been shown to increase in abundance with warming climate, and to modify the response of other plant species while doing so (Bråthen *et al.* 2017). Part of this effect is likely to be associated with the production of allelochemical metabolites, and therefore I hypothesize that metabolic production varies according to weather conditions. This metabolic production is expected to affect allelopathy potential, and therefore an effect on antioxidant activity is expected. Finally, shoot length is hypothesize to be affected by weather, as higher temperatures are expected to improve growth, and by metabolite production, as there can be trade-offs in the use of energy for growth and for chemical production.

Material and methods

Study region

Five locations in Northern Norway were chosen for this study, following a geographic and climatic gradient, from ocean to inland: Rebbenes (70.02N, 18.76E), Skogsfjord (69.95N, 19.25E), Snarby (69.76N, 19.51E), Skibotn (69.24N, 20.50E) and Kilpis (69.18N, 20.70E).

The study sites are located in the ecotone between the birch forest and open tundra, at the southern limit of the climatic subzone E (Walker *et al.* 2005), with mean July temperatures of 9-12 ºC. The zonal vegetation is dominated mainly by *Empetrum nigrum* L., which forms an open tundra from oceanic to more continental areas together with other dwarf shrubs <40 cm tall: *Betula nana* L., *Vaccinium uliginosum* ssp. *microphyllum* Lange, *Vaccinium vitis-idaea* L. (Pan Arctic Flora). . Thick moss carpets are common (*Hylocomium splendens* (Hedw.) Schimp., *Sphagnum*), and along drainages and near the treeline low and tall willows (*Salix glauca* L.) and alders are abundant.

Rebbenes is located in the inner part of Rebbenesøya (an island by the Norwegian Sea) in the vicinity of the shoreline, facing the continent (aspect east). Skogsfjord is located in Ringvassøy, an island east of Rebbenesøya, towards the mainland. This site is located farther from the coast and at a higher altitude, and presents a western aspect. Snarby is located in the continental area, following a near straight line from the previous two sites, in an area surrounded by fjords. It is located on the slope of a hill, facing east. Skibotn is in the mainland, far from sea and fjords, on a flat hillside, with south-east orientation. Kilpis is further inland than Skibotn, nearby the border between Norway and Finland, with east-northeast orientation. Rebbenes is the warmest site, with an average of 3.95 ºC over the last 60 years, and the wettest, with an average precipitation of 1004.4 mm (Table 1). The sites follow a decreasing trend in temperature and precipitation as they are more inland, with Skibotn and Kilpis showing similar conditions. The opposite pattern arises in average days of snow covered ground per year: Kilpis has the highest value (224.2), then a small decreasing trend until Skogsfjord, and finally a big difference from this to Rebbenes. Moreover, the variation of these variables also seem to follow a pattern, with smallest variation in temperature in the most coastal sites, while the smallest variation in precipitation and snow cover days happens in the inland sites (Table 1).

Study design

10 replicates of 3 permanent plots with a minimum of 5 meter distance between replicates and 0.5 meters between plots were established in 2009. During 7 years, from 2010 to 2016, 5 current year shoots of *Empetrum nigrum* were randomly selected from each of the three

permanent plots and the shoot length was measured. Samples were then taken to the lab, where they were dried at 50 ºC for 24 hours and then stored at room temperature in a dry place until processing. 3 out of the 10 replicates per site were chosen for the analysis, for a total of 15 samples per site and year, 105 samples for the 7 year period.

Chemical extraction

All leaves of all shoots were put into Eppendorf tubes for storage. 50±0.1 mg of leaves of each sample was then grinded into powder using a tissuelyser (Schwinglmühle TissueLyser 2, company *QIAGEN GmbH*). In order to do so the plant material and two stainless steel beads (5 mm diameter) were added to Eppendorf tubes, and the tubes were put in the tissulyser using a 24 tubes adaptor set. The tissuelyser was set at 22 rpm for 5 minutes, 3 times.

The extraction followed the method presented by Salminen and Karonen (2011). The resulting powder was extracted in 1.2 ml 80% aqueous shaking for two hours. The extracts were centrifuged for 5 minutes at 13000 rpm (Centrifuge 5415 R, company *eppendorf*), and 1 ml of the supernatant was transferred into new Eppendor tubes for chemical analysis. The pellet was resuspended in 0.5 ml 80% aqueous methanol for a second extraction. The samples were shaken again for two hours, and a second centrifugation was done. 0.5 ml of the supernatant were transferred to the Eppendorf tubes, and these carried to Marbio. 50 µl were pipetted into new Eppendorf tubes, in order to carry out a subsequent analysis of antioxidant activity.

Analysis of chemical composition

In order to measure leaf concentration of common secondary metabolites in *Empetrum*, samples were analysed for batatasin-III and caffeic acid. Samples were analysed at Marbio, a platform at the University of Tromsø for screening, isolation and identification of bioactive natural products and molecules. Analysis were carried out with an established protocol using UHPLC (Waters Aquity I-class), UV detection (Waters Aquity PDA) and high resolution mass spectrometry (Waters Vion IMS OTof). Samples were transferred to UHPLC vials and these placed in the injector in the UHPLC. 3µl of sample were injected on a Water Aquity BEH column (2.1x100 mm, 1.7 µm), and the sample components were eluted using a gradient of MilliQ H_2O (A) and acetonitrile (LC-MS Chromasolv Fluka)(B); both containing 0.1% formic acid. The mobile phase flow was 0.45 ml/min, and the gradient started at 95% A and 5 % B, and changed to 100% B over 12 min, holding at 100% B for 1.5 min.

The sample components were separated by the UPHLC and detected first by the UV-detector before they entered the mass spectrometer, where they were ionized by positive electrospray ionization. Mass data from 50 to 2000 Da were acquired at a scan time of 0.2 s. Capillary and

cone voltages were set at 0.8 kV and 30 V, respectively, while source and desolvation temperatures were set to 120 and 450 ºC, respectively. Collision energy ramped from 15 to 45 eV, and nitrogen (N_2) was used as cone and desolvation gas, at 50 and 800 L/h, respectively. The MS was set to high definition MS^E mode, and leucine-enkephalin (100 pg/µl) was infused as lock mass. The system was controlled by and data were analysed using the UNIFI 1.8.1application manager (Waters).

Purified samples of batatasin-III and caffeic acid were also run through the system, which gave information about these molecules' UV-spectrum. This allowed an easy identification of the compounds in the plant samples.

Antioxidant activity analysis

Measurements of antioxidant activity were performed by following the method presented by Jøraholmen *et al.* (2015). The method was adjusted to the current setting following suggestions by Basnet (personal communication) were performed. Antioxidant activity of leaf extracts was measured by DPPH (2,2-Diphenyl-1-picrylhydrazyl) method, with this substance as redox indicator, as it changes colour depending on the red-ox conditions. An original working solution of DPPH (120 µM) was made by dissolving 9.48 mg of DPPF into 200 ml of methanol, and optical density was adjusted to values between 0.5 and 0.6

Vitamin C ((2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one) was used as standard component and reference for our samples. Stock solution of Vit C (10 mg/ml) was prepared by dissoving it in water. The stock solution was then diluted a proportion 1:100 with ethanol. This process was repeated standard vitamin C solutions of 2, 4, 6, 9, 12 and 15 µg/ml were prepared in order to establish the standard curve.

Samples were first diluted in a 1:40 rate by adding 1950 µl of methanol (80%) to the 50 µl of sample kept after the extractions. 150 µl sample and 150 µl methanol were mixed in an Eppendorf tube(3 replicates per sample), together with 300 µl of DPPH solution, and left to react for half an hour. Afterwards each sample was transferred into a quartz cuvette and the UV absorption measured in the spectrophotometer at 515 nm (no interference from other molecules). The antioxidant activity of the plant samples was expressed as decreased optical density of DPPH radicals and it was compared with standard vitamin C.

Average shoot length

The average length of all shoots was calculated for each sample (15 shoots from each replicate). Shoot length is related to overall environmental and biotic conditions, as growth is

expected to be limited under stressful conditions (energy consigned to production of secondary metabolites).

Weather data

Temperature loggers were set over the soil surface, within the vegetation layer, in order to get a better idea of which conditions the plant is experiencing, with great importance of winter events. Joining together these two datasets date of snow disappearance could be determined. This marks the beginning of spring and plant growth and allows the calculation of the average temperature and precipitation until sampling day, and the computation of GDD (growing degree days). The formula, as presented by MCMaster and Wilhem (1997):

$$GDD = \frac{T_{max} + T_{min}}{2} - T_{base}$$

When GDD gave a negative value, i.e. $(T_{MAX}+T_{MIN})/2 < T_{BASE}$, then $(T_{MAX}+T_{MIN})/2$ was set equal to T_{BASE}, which gives 0 growing degree days (method 1 in McMaster and Wilhem 1997).

Additionally, from soil temperature data recurrence of extreme winter events was assessed, namely, periods in which soil surface temperature dropped far below 0 ºC and the ground froze. This indicates an absence of snow cover, or that, even present, it is not protective enough, and therefore an interval in which plants are exposed to extremely cold temperatures and might be damaged. Number of days where ground level average temperature was below -1 ºC (a conservative measure, days with temperatures close to 0 might still have water in liquid state) was calculated from one sampling period to the next one.

Data on air temperature at a resolution of 1 km^2 was obtained from senorge.no, in addition to data on precipitation and snow coverage.

Statistical analysis

All data were analysed using the statistical package R (R-3.4.2, Core Team 2017, https://www.R-project.org/). Four response variables were tested to see how they varied between sites: antioxidant activity, shoot length, batatasin-III and caffeic acid. Three samples of a total of 105 were removed from the analysis on antioxidant activity, one of them due to a measurement error in antioxidant activity, as it showed a negative value; the remaining two were removed due to their impact on the distribution, and were shown to be outliers in a boxplot.

Distribution of batatasin-III and caffeic acid was not normal (Shapiro-Wilk<0.001), with a big peak for the smallest values and a long, flat tail afterwards. Distribution of antioxidant activity did not differ from normality after removing the outliers, and neither did distribution of average shoot length after applying logarithmic transformation.

One-Way ANOVA was used to detect site differences in antioxidant activity and average shoot length, followed by a pairwise comparison using Tukey's honest significance difference test, which also applies a correction for multiple comparisons. For the not normally distributed variables, batatasin-III and caffeic acid, non-parametric tests were used: Kruskal-Wallis test to see if sites differed, and Dunn's test for post-hoc analysis.

The site specific weather data were analysed using non-metric multidimensional scaling (NMDS) according to the formula in *metaMDS* (package *vegan*, Oksanen *et al.* 2017). NMDS is an ordination method that collapses information from multiple dimensions into just a few, so that they can easily be visualized and interpreted. It uses rank orders, and thus is an extremely flexible technique that can accommodate a variety of data distributions (Oksanen 2015).

It begins with a distance matrix of all points in the multi-dimensional space, and sets (compresses) all points into a random position in the required dimensions. Afterwards, it applies the function *monoMDS*, an algorithm that moves points around until distances in the new dimensions go in the same order (rank) as distances in the original space. Difference between the original distances and the new created ones is called stress (measurement of goodness of fit). After one try the solution reached might be a local optimum and not the global one. *metaMDS* overcomes this by applying *isoMDS* several times with different random starts. The function then rotates the solution so that the variance of points is maximized on first dimension.

The *rankindex* function was used in order to choose a dissimilarity matrix, as it establishes rank correlations between dissimilarity indices and gradient separation. Several dissimilarity indices gave the same result using he *rankindex* function, so the Bray-Curtis index was chosen due to the fact that, even though it is meant for community composition differences (based on counts), it performs well at detecting underlying ecological gradients (Faith *et al.* 1987). Ordinations were run with several dimensions, and the ordination with the fewest dimensions and a stress value close to or below one was selected (Lefcheck 2012) and that number of dimensions used. Soil temperature data was available only from 2012 to 2016, so two ordinations were performed: one with samples from all years but without soil-related variables, and another one with all climatic variables, but samples only from 2012 to 2016.

Plant traits (secondary metabolites, antioxidant activity and shoot length) were fitted onto the ordination by means of the *envfit* function. This function calculated the correlation values of each plant variable with the ordination space, and allowed to display the plant variables on the ordination plot. The *vf* function from the package *ecodist* (Goslee and Urban 2007) was applied to calculate the correlation between each weather variable and the dimensions and with the ordination space. Anosim and Adonis were used to determine whether sites differed or not, and,

as no post-hoc analysis is available, pairwise adonis tests were performed to determine which sites are dissimilar and values were adjusted by bonferroni's correction for multiple comparisons.

Variables with the highest correlation in the ordination analyses were chosen for modelling. However, as yearly air temperature and number of freezing days were highly correlated (-0.695), and the latter was available for only a subset of the years (2012-2016), number of freezing days was discarded from the posterior analyses. Plant traits were analysed according to the axis that explained most of their variation.

In the case of leaf concentration of batatasin-III, average shoot length and yearly air temperature, general relations were checked by using Kendall-Theil Sen Siegel test. Much of the variation was represented by sites, which suggested within site variability could be partly responsible for variation in these variables. Therefore, each variable was scaled at each site at an average of 0 and a standard deviation of 1, which allowed to use parametric modelling. These variables were analysed using linear mixed models, fitted using the *lme* function from the package *nlme* (Pinheiro *et al.* 2017). In the first model, leaf concentration of batatasin-III was set as response factor, yearly air temperature and site as fixed predictors (with interaction) and sampling plot and year as random factors. In the second model, average shoot length was set as response factor, concentration of batatasin-III and site as fixed predictors (with interaction) and sampling plot and year as random factors. In the third model, average shoot length was set as response factor, yearly air temperature and site as fixed predictors (with interaction) and sampling plot and year as random factors.

In the case of leaf concentration of caffeic acid, antioxidant activity and summer precipitation the distribution was problematic, so no parametric tests could be performed. The Kendall-Theil Sen Siegel test was the only model used to fit these variables. In the first model concentration of caffeic acid was modelled according to summer precipitation; in the second antioxidant activity was modelled according to caffeic acid; and in the third antioxidant activity was modelled according to summer precipitation.

Results

Batatasin-III and caffeic acid concentration

The concentration of batatasin-III was high in Skibotn and Kilpis (51.5 and 65.4 mg/g leaf dry matter), intermediate in Rebbenes (32.5 mg/g), low in Snarby (15.3 mg/g) and extremely low in Skogsfjord (1.6 mg/g) (Figure 1). Meanwhile, the content of caffeic acid was quite variable within site and year, with values from 0.9 to1.9 mg/g leaf dry matter (Figure 2). Sixteen samples contained no detectable caffeic acid (n=105), with no clear pattern by site or year.

The Kruskal-Wallis test on caffeic acid did not give any indication of site differences (chi-squared: 2.68; d.f.=4; p=0.61), even when removing 0s from the analysis (chi-squared=5.15; d.f.=4; p=0.27). It did, however, show site differences for batatasin-III (Kruskal-Wallis chi-squared:64.92; d.f.=4; p<0.001).

Antioxidant activity and shoot length

Values of antioxidant activity ranged from 2.83 to 3.08 mg Vitamin C equivalent / 50 mg leaf dry matter (Figure 3). The ANOVA test suggested there were some site differences (F=2.71; d.f.=4, 97; p=0.035), but the post-hoc analysis (Tukey's HSD test) did not show any significant differences. Average shoot length varied from 0.93 cm to 1.66 cm. and the ANOVA test gave significant result (F=25.87; d.f.=4, 100; p<0.001) (Figure 4).

Ordination analysis

The ordination with 2 dimensions gave a stress value of 0.120, so it was the one chosen as the most parsimonious ordination. Yearly air temperature (YAT) was the main variable determining the first axis (Figure 5 and Table 2), and hence the one more highly correlated with the ordination space, meaning it was the variable best explaining differences between samples. The rest of the variables were close to the origin of coordinates, with small NMDS1 and intermediate to small values of NMDS2, meaning they contributed to the second axis. Out of these variables, summer precipitation was the one with the highest value (Table 2).

Average shoot length was highly correlated with the first axis, with high values of NMDS1 and small of NMDS2 (Figure 5). Caffeic acid and antioxidant activity were both mainly related to the second axis, with antioxidant activity more highly correlated than caffeic acid (Table 2). Batatasin-III had large values in both axes, meaning it was affected mainly by NMDS1 but NMDS explain also part of the variability.

Sites were located in the ordination plot following the climatic gradient, with warmer sites to the left of the ordination plot (Figure 6. Same ordination space provided by the weather variables, but now displayed in their relation to the sites and their samples). Rebbenes, Skogsfjord and Snarby were close together, with a certain degree of overlap, and had a great variation in the second axis and small in the first one. Skibotn and Kilpis, on the other hand, overlapped extensively and had a great variation in both axes (Figure 6).

Sites were significantly different, as both anosim and adonis gave a p value of 0.001. Skibotn and Kilpis are similar between them and different from all the other sites (Table 3). Snarby is similar to Skogsfjord but different from Rebbenes; and these last two sites are close to dissimilarity (p values ranging from 0.033 to 0.073).

The second ordination analysis, using samples from 2012 to 2016 and taking into account all weather variables, gave similar results. The main difference was that number of freezing days turned out to be important.

Effects of weather variables on plant traits

Based on the NMDS analysis, three weather variables were selected: year air temperature, summer precipitation and number of freezing days during winter. However, the latter was highly correlated with yearly air temperature (r=-0.695), so it was discarded from the modelling, as we would not be able to separate effects due to number of freezing days from effects due to yearly air temperature.

The ordination that batatasin- III, average soot length and yearly air temperature varied mainly in the first axis. There was a negative relation between yearly air temperature and batatasin-III, and a positive with average shoot length. These relations were analysed using the Kendall-Theil Sen Siegel test (non-parametric linear regression). The lower the yearly air temperature, the higher the production of batatasin-III and the shorter the shoots (Table 4). However, the lme model pointed out that there is a positive within site effect of temperature on production of batatasin-III, with no site specific differences (Table 5, Figure 7). No relation between shoot length and batatasin-III or yearly air temperature was found in the linear mixed models (Table 6).

The ordination showed that caffeic acid, antioxidant activity and summer precipitation varied mainly along the second axis. The Kendall-Theil Sen Siegel test showed positive but weak relations between the three variables (Table 7).

Discussion

While concentration of batatasin-III and average shoot length changed along the climatic gradient, confirming our hypothesis, no change in antioxidant activity was found, against what we hypothesized. As hypothesized, concentration of batatasin-III was also affected by weather conditions at the sited level. Meanwhile, shoot length was not affected by weather condions or metabolic production, against our hypothesis. Antioxidant activity, caffeic acid and summer precipitation were positively related, but it was not possible to analyse them at the site level, so the hypothesis could not be tested.

Effects of climate have already been found to affect concentration of plant defences in oaks (Pearse and Hipp 2012), and in other allelochemical species, like *Cistus ladanifer* (Lobón *et al.* 2002). In this study, 11 allelochemicals of *Cistus ladanifer* were found to respond

heterogeneously to temperature and photoperiod, but to respond positively when studied conjointly. In comparison to previous studies, we also found climate to affect the production of an allelochemical compound.

Site differences and effects of weather on concentration of batatasin-III and caffeic acid

This is, to our knowledge, the first study attempting to quantify batatasin-III production in sites with different climatic conditions. Nilsson *et al.* (1998) established a study where she followed the production of phenolics and batatasin-III by *E.nigrum* ssp. *hermaphroditum* throughout several years. The study was conducted in a single site, and managed to find patterns in the seasonal production, and according to the age of shoots, but failed to linked year-to-year variation to the weather factors. As she phrased it, *climatic effects on long-lived plants are generally difficult to detect over time spans of a few years*. In our study, however, we took samples from sites with quite different weather regimes, addressing both intra-site and inter-site variation to give us some clues about climate effect on production of allelopathic compounds.

Batatasin-III had its highest values in the most continental sites. In these sites plants are exposed to lower temperatures all year around (0.029±0.85 ºC and 0.10±0.83ºC, respectively), and are under a thick and continuous layer of snow during most of the winter (Figure 8), despite having less precipitation (401.7±65.6mm and 395.5±44.2mm, respectively). The snow cover constitutes a protecting layer during winter: isolates the plants and soil from the cold air and windy conditions, giving plants a warmer environment (less winter hardening required) and keeps soil from freezing (Vikhamar-Schuler *et al.* 2016). However, it seems it is not enough to protect the soil in Skibotn and Kilpis, as is reflected by the number of freezing days per year (120.8±73.6 and 152.8±22.9, respectively). Our results are in accordance with previous studies, as allelopathy has been highlighted as important in stressful environments (Inderjit and Del Moral 1997, Wardle *et al.* 1998), and batatasin-III has been pointed out as the main metabolite responsible for allelopathic effects by *Empetrum nigrum* (Odén *et al.* 1992, Nilsson *et al.* 1998, González *et al.* 2015).

Rebbenes, though having the warmest yearly temperature (4.55±0.42ºC), has an intermediate value of batatasin-III. This could be due to the fact that precipitation in winter falls both as rain and snow, and the little snow that accumulates melts easily (with several and long periods of snow-free ground), which suggests that plants are more easily exposed to freezing temperatures (41.2±17.5 freezing days per year, big variation), and rain on snow events would be expected to be more common.

Despite the fact that plants in colder sites seem to produce more batatasin-III, a within-site increase in production with temperature was found. This is significant in the current global

context, as there has been an expansion of dwarf shrub species throughout the Arctic (Sturm *et al.* 2001, Tape *et al.* 2006), and global warming has been proposed as the most likely cause for this phenomenon (Serreze *et al.* 2000, Elmendorf *et al.* 2012). Analysing the temperature data from the last 60 years, an increasing trend in average year temperature was also found in the current study, with an increase of 0.024ºC per year (0.013-0.036: 95% confidence interval. Figure 9). Warmer temperatures can lead to a higher metabolic production, but also may allow the advancement of other dwarf shrubs coming from the south, like *Calluna vulgaris*, which would increase the competition. González et al. (2015) demonstrated that batatasin-III inhibits germination and mean root elongation in *Calluna* already at very low concentrations (0.0-0.5mM gave 50% inhibition). Hence an increase in production of batatasin-III could be expected under more competitive conditions.

Caffeic acid, meanwhile, showed high within site variability, leading to no site differences ($p=0.61$). It was related to summer precipitation, but only in a loose way. Caffeic acid is a key intermediate in the production of lignin (Boerjan *et al.* 2003), and therefore it is likely to have a high production at all sites. Moreover, caffeic acid, as well as many other phenolic acids, is degraded progressively in leaves, producing, protocatechuic acid (Gallet *et al.* 1999), which has been shown to enhance antioxidant activity (Rice-Evans *et al.* 1996), and to reduce hydraulic conductivity and nutrient uptake from roots (Blum 1996). Further research should consider the effect of climate on protocatechuic acid, and for that the focus should be given to phenolic acids in general (degrading into protocatechuic acid) and to older shoots (as the highest concentration of protocatechuic acid is found in brown leaves, Gallet *et al.* 1999).

Concentrations of both metabolites were generally higher (taking into account the high variability displayed by batatasin-III) than those obtained by Gallet *et al.* (1999). However, they took into account leaves of both the first and second year, while in the present study only first year shoots were included. Batatasin-III has been shown to reach its maximum at the end of the first summer, with a small decrease during the second year (Nilsson *et al.* 1998). And caffeic acid is degraded progressively in leaves, producing protocatechuic acid (Gallet *et al.* 1999). These two ideas suggest that concentration of both compounds in the present study was near its zenith at the moment of sampling.

Site differences and effects of weather on antioxidant activity and shoot growth

Antioxidant activity was used intended to be used as a proxy of allelopathic activity. However, antioxidant activity did not vary between sites, and the ordination analysis gave no indication of it being related to batatasin-III, only to caffeic acid. While batatasin-III has been shown to have direct inhibitory effects (e.g. disturbing proton pumping in plasma membranes, Wallstedt 2001; inhibiting CO2-dependent oxygen evolution and electron flow from water to

acceptors, Iino 1978), antioxidant activity is a more indirect phenomena, affecting the oxidative state of phenolic compounds.

As expected, plants in the sites with the lowest temperatures had the shortest shoots. Note that Snarby, despite having the shortest growing season (84.0±2.7 days), had the longest shoots, which suggests length of growing season was not important for shoot growth. The continuous and thick layer of snow present in Snarby during winter makes it protect from freezing events, which is also shown in the very low number of freezing days (6.8±2.9, the lowest of all sites). Nevertheless, *E. nigrum* has a very conservative growth strategy, and it reacts slowly to environmental changes (Tybirk *et al.* 2000). This is shown in our linear mixed model, as temperature variation within site had no significant effect on average shoot length.

A higher allocation of energy and nutrients to production of secondary metabolites can be costly and lead to a reduced growth (Moore et al. 2014). In the present study no such relation was found, as average shoot length did not vary according to the concentration of batatasin-III or caffeic acid. Nonetheless, only two compounds were taken into account. A broader array of metabolites should then be studied, using methods that allow the determination of wide metabolite families, e.g., studying the whole phenolic production.

In conclusion, even though plants from colder sites had a higher concentration of batatasin-III in their leaves, increase in temperature at each site seems to increase the production of batatasin-III. This could help us understand how *Empetrum nigrum* may respond to climate change and gain dominance. Further research will be needed to assess the impact of this increase at the ecosystem level. No site differences were found in caffeic acid, which could be due to its importance also as an intermediate molecule, as in the biosynthesis of lignin. No relation between secondary metabolite production and antioxidant activity was found, which suggests this is affected by other molecules (e.g., phenolic acids). And neither did concentration of secondary metabolites relate to shoot length. These two ideas suggest that more compounds should be look into: many metabolites exhibit antioxidant activity, and a high total production might be linked to a decrease in allocation of nutrients to growth.

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Figures

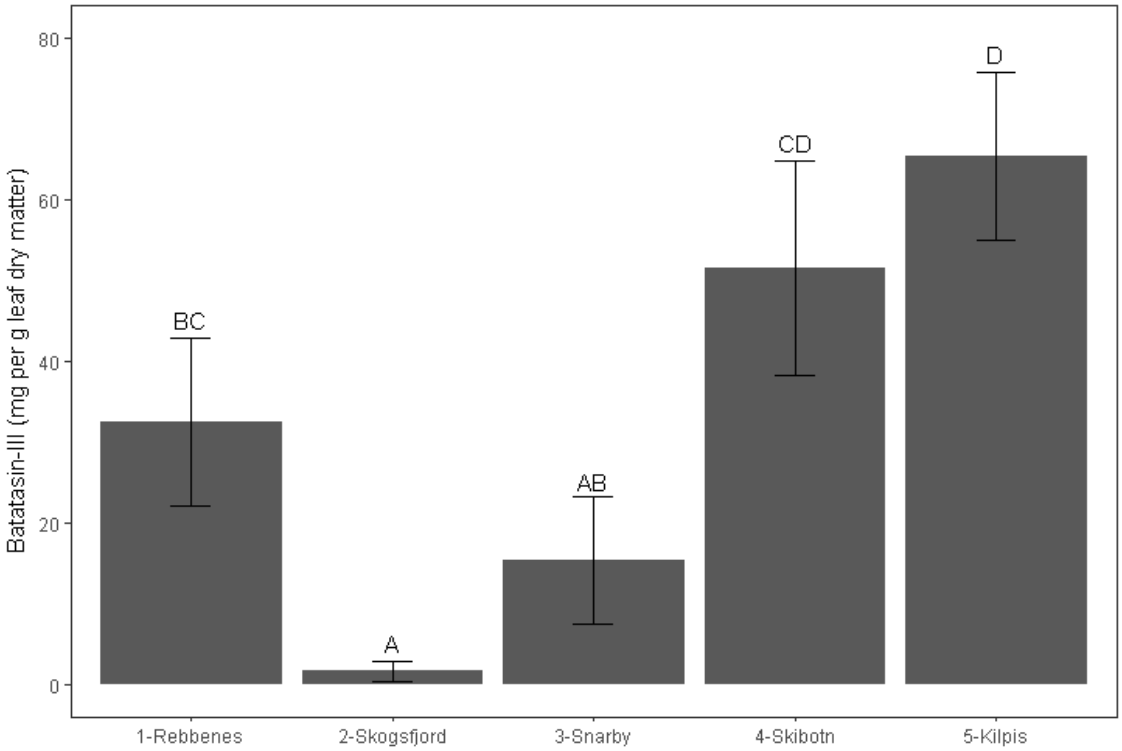

Figure 1. Average concentration of batatasin-III in mg/g leaf dry matter across 7 years at each site. Error bars show 95% confidence intervals. Bars with same letter are not different at a 95% significance level (Dunn's test).

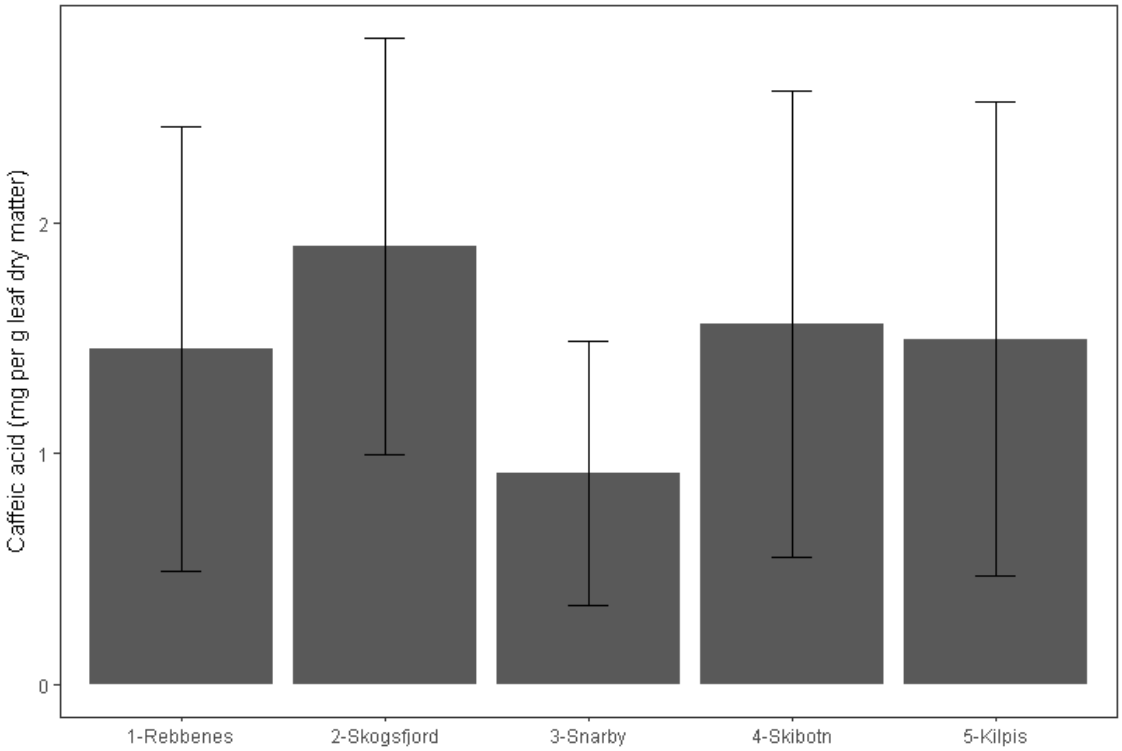

Figure 2. Average concentration of caffeic acid in mg/g leaf dry matter across 7 years at each site. Error bars show 95% confidence intervals.

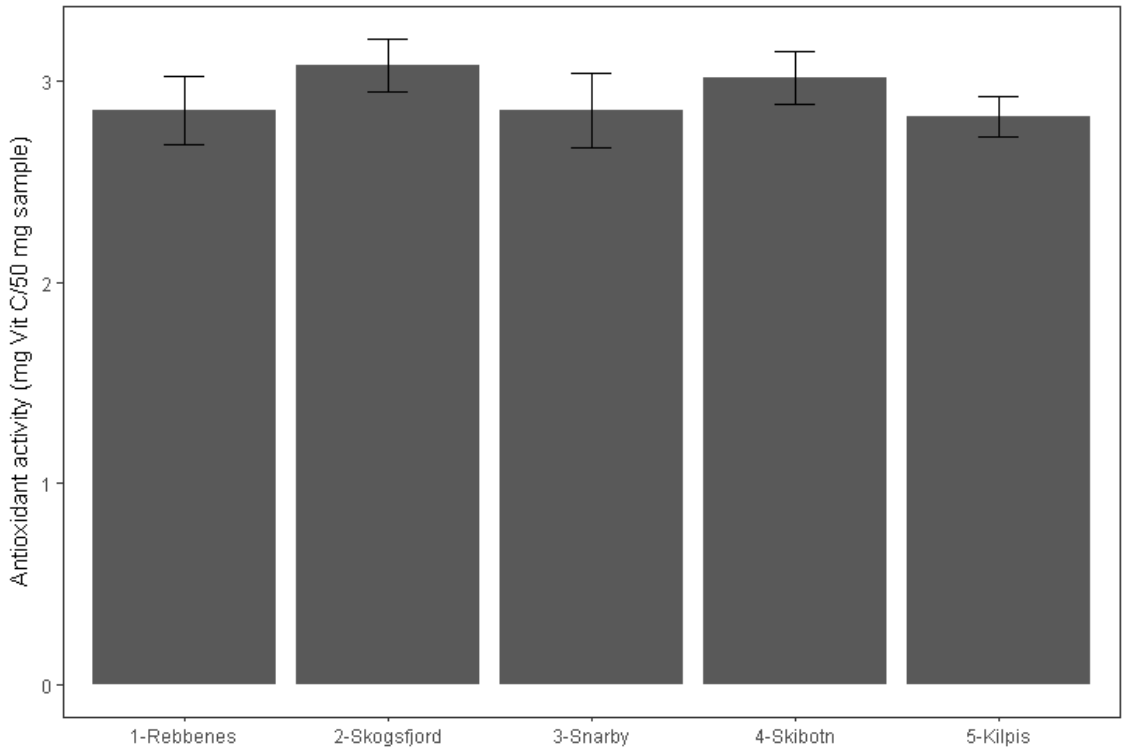

Figure 3. Antioxidant activity (in mg Vit C per 50 mg sample) per site across seven years. Error bars show 95% confidence intervals.

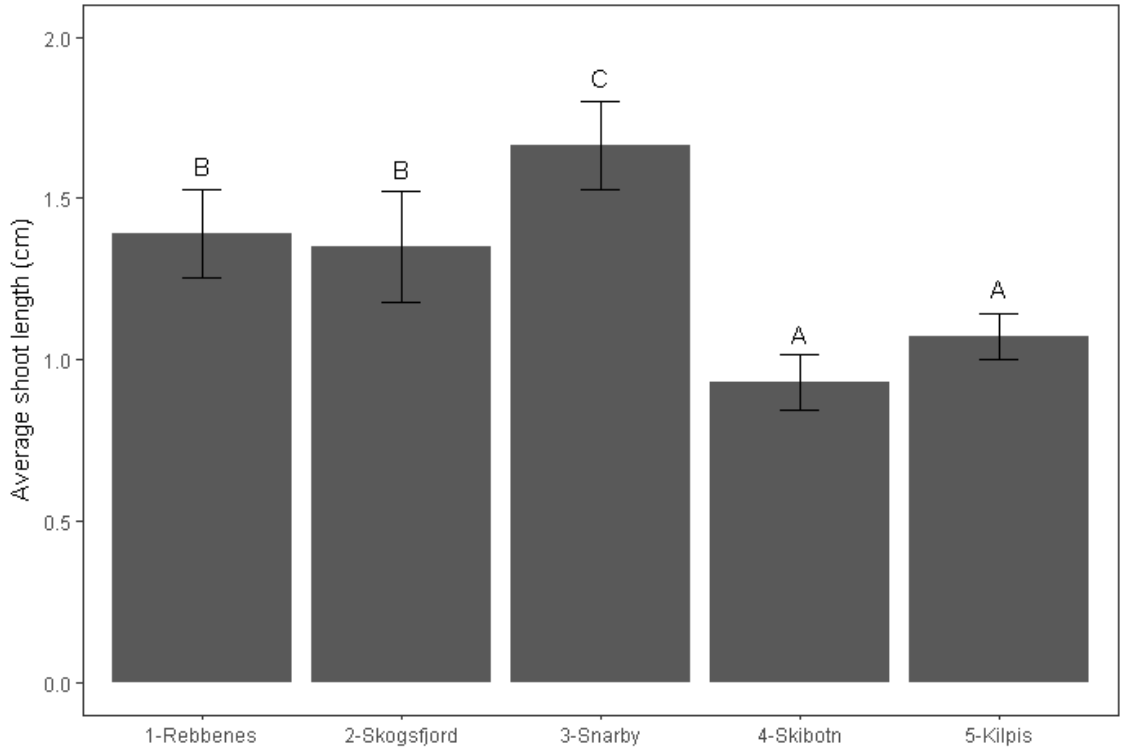

Figure 4.Average shoot length (in cm) per site across seven years. Error bars show 95% confidence intervals. Bars with same letter do not differ at a 95% significance level (Tukey's HSD test).

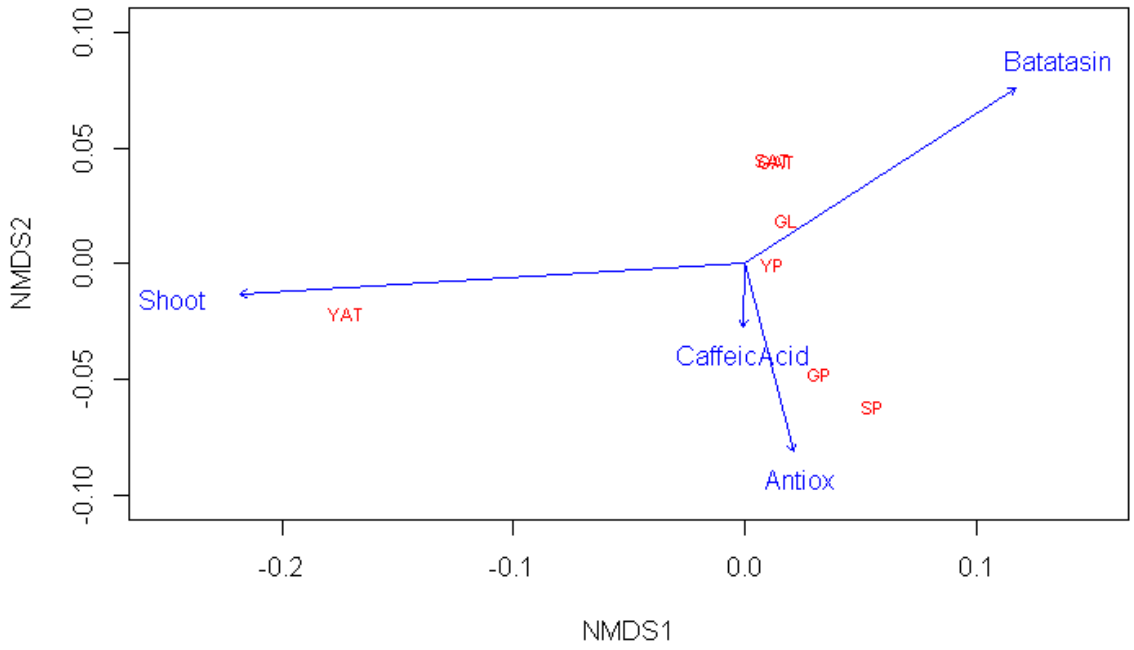

Figure 5. Ordination plot created by using climatic variables from 2010 to 2016, yearly air temperature (YAT), overlapping in the diagram with summer air temperature (SAT), air temperature during growing season (GAT), yearly precipitation (YP), summer precipitation (SP), precipitation during growing season. (GP) and length of growing season (GL).

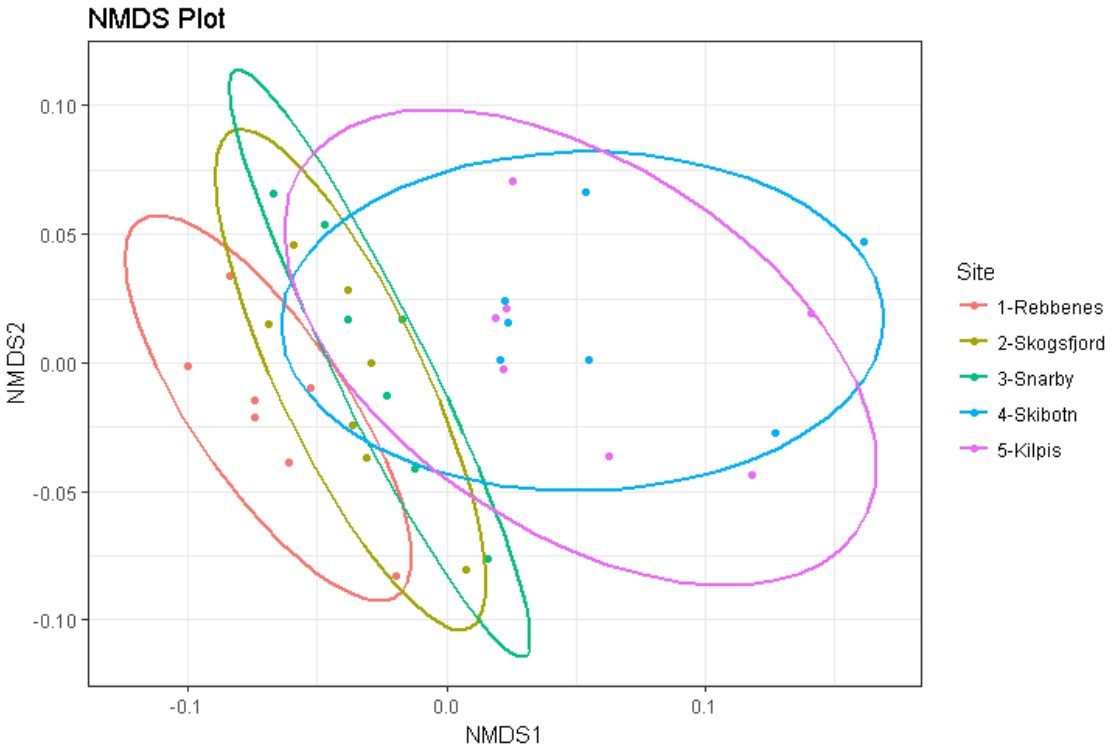

Figure 6. Ordination plot. Dots of different colours represent the 5 sites, and each dot represent one specific sample (each year at each site). Ellipses encompass a 95% confidence interval for each site.

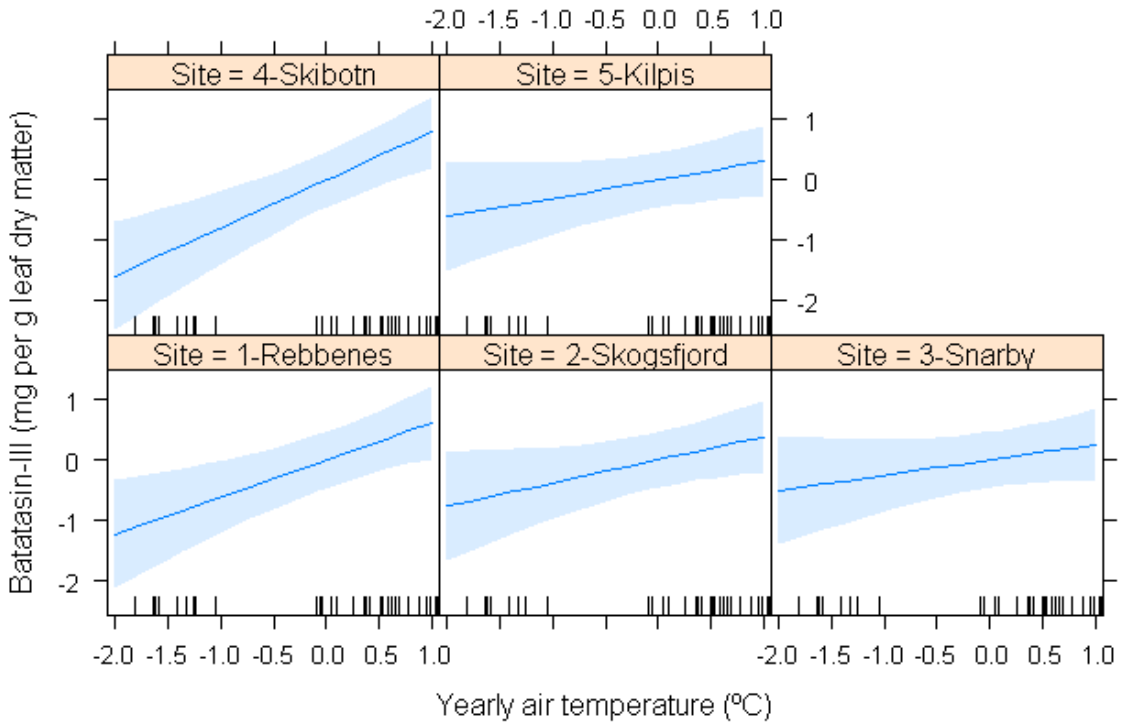

Figure 7. Site-wise effect of yearly air temperature on production of batatasin-III. Both variables are scaled at the site level. Trend and 95% confidence interval are depicted.

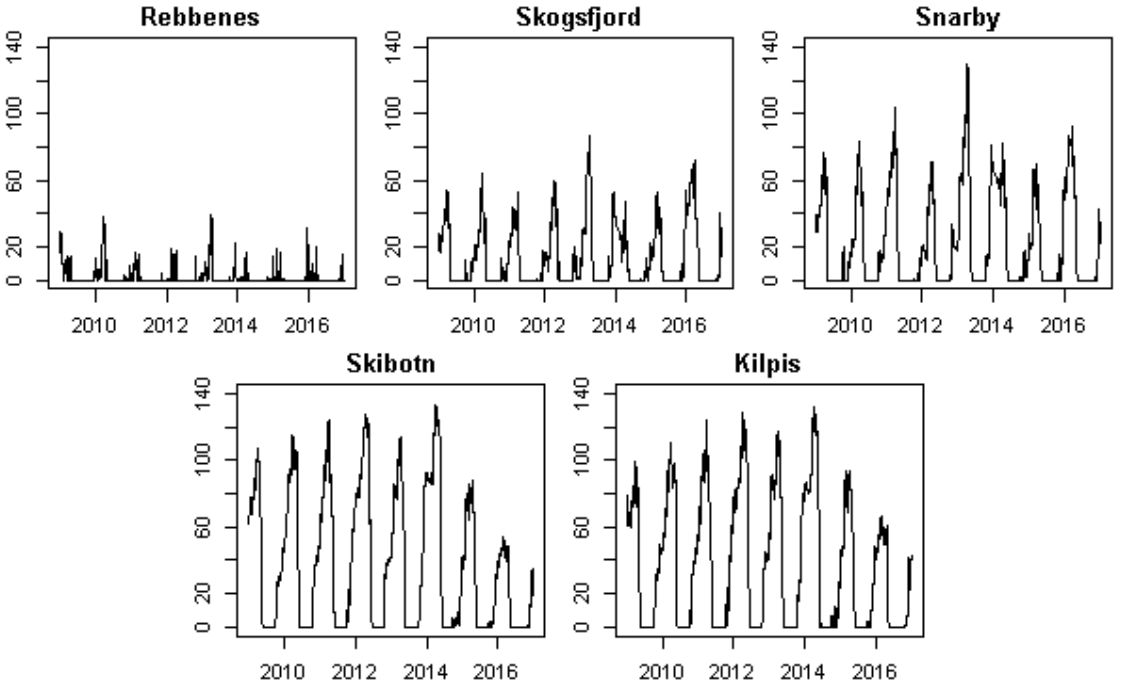

Figure 8. Thickness of snow layer (in cm) during the study period at each of the study sites. Data acquired from senorge.no.

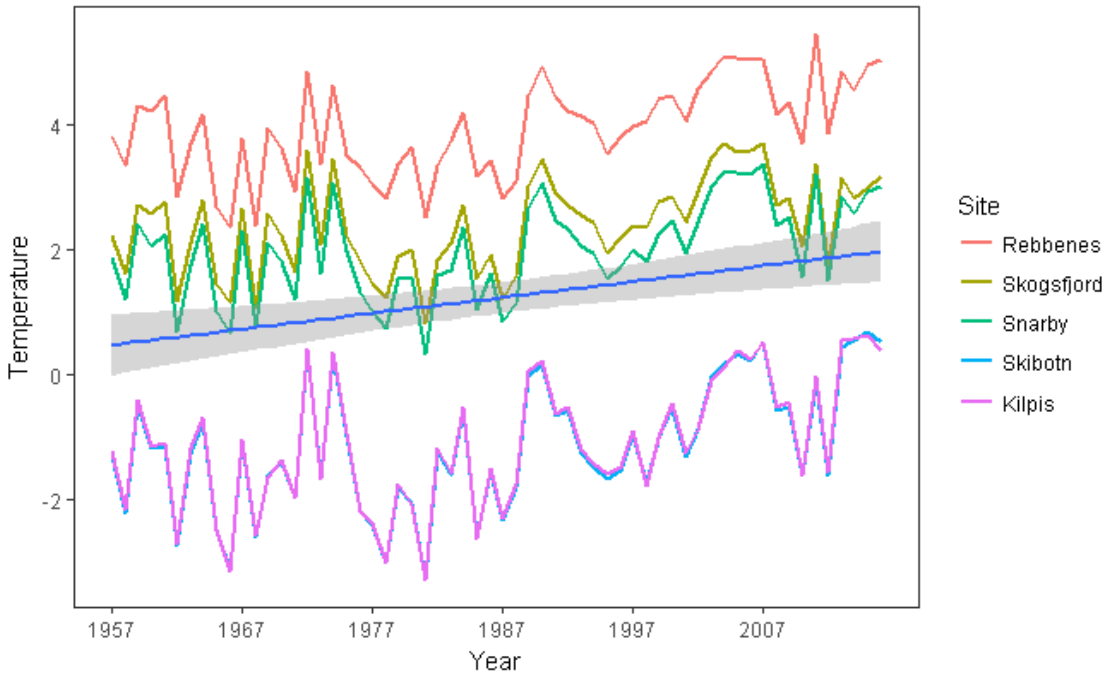

Figure 9. Average year temperature from 1957 to 2016 by site: Rebbenes, Skogsfjord, Snarby, Skibotn and Kilpis. Global trend is presented with 95% confidence interval (shadowed area). Data obtained from senorge.no.

Tables

Table 1. Average values of temperature, precipitation and days with snow cover for each site. 95% confidence intervals are provided. Data from senorge.no

	Temperature (ºC)	Precipitation (mm)	Snow cover days
Rebbenes	3.95 ± 0.20	1004.4 ± 47.2	120.3 ± 11.4
Skogsfjord	2.40 ± 0.19	990.2 ± 48.0	187.8 ± 6.8
Snarby	2.01 ± 0.20	931.8 ± 46.9	194.5 ± 6.1
Skibotn	-1.14 ± 0.27	545.6 ± 41.8	216.2 ± 5.1
Kilpis	-1.11 ± 0.27	531.9 ± 35.0	215.5 ± 4.6

Table 2. Upper section: values and correlation of each climatic variable with the dimensions (r) and with the ordination space (R). Lower section: correlation of the plant traits with each dimension (r) and with the ordination space (R) (calculated by *vf* function from package *ecodist*).

	NMDS1		NMDS2		
	Value	r	Value	r	R
Year air temperature	-0173	-0.941	-0.021	-0.338	0.955
Summer air temperature	0.013	-0.815	0.045	0.580	0.490
Growing air temperature	0.014	-0.798	0.045	0.603	0.366
Year precipitation	0.012	-0.248	-0.000	-0.969	0.574
Summer precipitation	0.056	0.249	-0.062	.0.968	0.889
Growing precipitation	0.033	0.084	-0.047	-0.996	0.787
Growing season length	0.018	-0.373	0.019	-0.928	0.381
Batatasin-III		0.839		0.544	0.352
Caffeic acid		-0.025		-1.000	0.069
Antioxidant activity		0.249		-0.968	0.211
Shoot length		-0.998		-0.059	0.550

Table 3. Adonis pairwise comparisons according to the sites' weather characteristics, adjusted by bonferroni's correction for multiple comparisons.

	Rebbenes	Skogsfjord	Snarby	Skibotn
Skogsfjord	0.63	–	–	–
Snarby	0.01	0.65	–	–
Skibotn	0.01	0.01	0.01	–
Kilpis	0.01	0.01	0.01	1.00

Table 4. Relation of average shoot length, batatasin-III and yearly air temperature. Associated probabilities and 95% confidence intervals are shown.

	Batatasin-III		Yearly air temperature	
	Effect	p value	Effect	p value
Average shoot length	-0.003 (-0.004 | -0.002)	<0.001	0.12 (0.10 | 0.13)	<0.001
Batatasin-III	–		-3.61 (-6.63 | -3.33)	<0.001

Table 5. Effect of yearly air temperature on batatasin-III production. Only the effect of the variable and the interaction terms are included.

Batatasin-III	Effect	P value
Yearly air temperature	0.61 (0.23 | 0.99)	0.002
YAT-Skogsfjord	-0.23 (-0.73 | 0.26)	0.353
YAT-Snarby	-0.36 (-0.85 | 0.14)	0.154
YAT-Skibotn	0.18 (-0.32 | 0.67)	0.475
YAT-Kilpis	-0.31 (-0.80 | 0.19)	0.221

Table 6. Effects of batatasin-III concentration and yearly air temperature on average shoot length. Only the effect of the variable and the interaction terms are included.

Average shoot length	Effect	P value
Batatasin-III	0.18 (-0.27 | 0.62)	0.432
Bat-Skogsfjord	-0.46 (-1.08 | 0.16)	0.141
Bat-Snarby	-0.14 (-0.76 | 0.47)	0.642
Bat-Skibotn	0.09 (-0.51 | 0.69)	0.763
Bat-Kilpis	-0.14 (-0.75 | 0.47)	0.652
Yearly air temperature	0.23 (-0.20 | 0.66)	0.283
YAT-Skogfjord	-0.33 (-0.92 | 0.27)	0.276
YAT-Snarby	0.21 (-0.38 | 0.80)	0.480
YAT-Skibotn	0.35 (-0.24 | 0.94)	0.245
YAT-Kilpis	-0.09 (-0.68 | 0.50)	0.759

Table 7. Relation ofantioxidant activity, caffeic acid and summer precipitation. Associated probabilities and 95% confidence intervals are shown.

	Caffeic acid		Summer precipitation	
	Effect	p value	Effect	p value
Antioxidant activity	0.102 (0.086 | 0.146)	<0.001	0.001 (0.001 | 0.002)	<0.001
Caffeic acid			0.001 (0.001 | 0.005)	<0.001