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Research Article

Intra-specific variability and sex influence allelopathic capacity of *Empetrum nigrum*

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Allelopathic plant species play significant roles in shaping natural ecosystems. While a high degree of inter-species variation in allelopathic capacities is commonly reported, variation across intra-specific groups and between dioecious sexes is limited. Here we use the ecologically important, allelopathic dwarf shrub *Empetrum nigrum* to assess how allelopathic capacity varies between intra-specific groups (i.e. the diploid and dioecious '*nigrum*' group and the tetraploid and synoecious '*hermaphroditum*' group of *Empetrum*) and sex (i.e. male and female plants of the *nigrum* group). Specifically, we tested Icelandic *Empetrum*, as its allelopathic nature may influence long-term land degradation across Icelandic highlands. The allelopathic capacity of *Empetrum* leaves was measured in a series of bioassays as the inhibition of seed germination and seedling root elongation of the grass *Festuca richardsonii*. A very strong allelopathic response of *Empetrum* leaves was observed. Intra-specific and sex-related differences in allelopathic capacity were also detected, with the *hermaphroditum* group possessing a greater germination inhibitory effect compared to the *nigrum* group, and males of the *nigrum* group inhibited germination more than females. Targeted liquid chromatography-mass spectrometry (LC-MS) quantification of two known *Empetrum* allelopathic constituents – Batatasin-III (BIII) and 3,4-dimethoxy-2',5-dihydroxybibenzyl (DDB) – showed that BIII concentrations were significantly higher in leaves from the *hermaphroditum* group compared to the *nigrum* group, but no differences were observed between males and females within the *nigrum* group. DDB was not detected in the sampled Icelandic *Empetrum* population. Our results indicate that *Empetrum* may differentially impact plant interactions and ecosystem processes depending on the relative abundance of *Empetrum* intra-specific groups and sexes within populations, and that BIII is not solely responsible for the allelopathic capacity of Icelandic *Empetrum*.

Keywords: allelopathy, crowberry, dioecious, *Empetrum hermaphroditum*, *Empetrum nigrum*, intra-specific

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Introduction

Allelopathy is the chemical interaction between plant species where one organism may promote or interfere with the other (defined by the International Allelopathy Society). However, allelopathy has often been used to refer to negative interference, whereby plant species release specialized metabolites (allelochemicals) that inhibit the growth and development of their neighbours (Kumar et al. 2024), and we will refer to allelopathy as such in our study. Allelopathic plant species therefore, play a significant role in natural ecosystems, affecting e.g. species distribution and diversity (Hierro and Callaway 2021). The impact of allelopathy on shaping plant communities may depend on intra-specific variability in the allelopathic capacity of different plant species. This is due in part to a tight link between genetic variation and allelopathy, which thereby varies between different taxonomic units (Muzell Trezzi et al. 2016). Considerable variation in allelopathic capacity between plant intra-specific groups has previously been documented. For example, Zou et al. (2007) documented differential allelopathic capacities between different wheat *Triticum aestivum* accessions and related the variation to genetic, biochemical, and environmental factors. Fewer studies, however, have addressed whether allelopathic capacity is affected by the sex of dioecious plant species. Sexual variation in allelopathic capacity may be expected due to differential resource allocation, where female plants may invest more in reproduction, leaving fewer resources available for other plant functions (Ågren et al. 1999), such as the production of allelochemicals. Indeed, in a stand-alone study into sex-related differences in allelopathic effects on neighbouring plants, Avendaño-González et al. (2016) reported that male individuals of the Peruvian peppertree *Schinus molle* were generally more allelopathic than females.

Empetrum nigrum is a strongly allelopathic evergreen dwarf shrub (Tybirk et al. 2000), which is widely distributed in the northern hemisphere (Büntgen et al. 2015). The allelopathic capacity of *Empetrum* has previously been demonstrated in bioassays for seed germination and seedling root elongation of several plant species (Nilsson and Zackrisson 1992, Bråthen et al. 2010, González et al. 2015, Pilsbacher et al. 2021), and in-field experiments assessing seedling establishment and survival (Nilsson and Zackrisson 1992, Nilsson 1994, Nilsson et al. 2002, González et al. 2021). Two major intra-specific *Empetrum* groups in the northern hemisphere are distinguished by their ploidy levels and reproductive morphology: one diploid and dioecious, and the other tetraploid and synoecious (Wasowicz 2020). These two groups have previously been recognized as *Empetrum nigrum* ssp. *nigrum* and *Empetrum nigrum* ssp. *hermaphroditum*, respectively. However, the taxonomic status of these groups is more complicated than that (Murray et al. 2009, Elven et al. 2011), as tetraploids appear to have arisen multiple times from various combinations of diploids, making their assignment to a single lineage or taxon challenging (Elven et al. 2011). Therefore, to avoid assigning an incorrect taxonomical status to these intra-specific *Empetrum* groups, we refer to them as

the ‘*nigrum* group’ and ‘*hermaphroditum* group’, respectively, in our study.

Intra-specific variability in *Empetrum* allelopathic capacities has previously been documented. For example, Nilsson et al. (2000) observed that the *hermaphroditum* group has stronger allelopathic capacities than the *nigrum* group in northern Sweden. Furthermore, Valsdóttir (2021) observed allelopathic differences between two *Empetrum* populations in Iceland, which may be linked to variation in the abundance of the *hermaphroditum* and *nigrum* groups. The allelopathic capacity of Scandinavian-based *Empetrum* intra-specific groups has been largely attributed to qualitative and quantitative variation in bibenzyl level (Nilsson et al. 2000), where Batatasin-III (herein referred to as BIII) is a major bibenzyl in leaves from the *hermaphroditum* group (Oden et al. 1992) and 3,4-Dimethoxy-2',5-dihydroxybibenzyl (herein referred to as DDB) is present in leaves from the *nigrum* group (Jarevång et al. 1998). It is currently unknown if Icelandic *Empetrum* populations possess similar levels of BIII and DDB as is found in Scandinavia, for example. Furthermore, sex-related differences in the allelopathic capacity of *Empetrum* has not been performed.

Here we aim to investigate differences in the allelopathic capacity of different Icelandic *Empetrum* intra-specific groups and sexes. Both the *nigrum* and *hermaphroditum* groups are abundant in Iceland (Wasowicz 2020), particularly in degraded tundra ecosystems (Jónsdóttir et al. 2005), where *Empetrum* likely plays a role in retarding ecosystem transitions to more productive states (Ryde et al. 2025a). Knowledge of how allelopathic capacity varies among *Empetrum* intra-specific groups and sexes, could enhance our understanding of how the role of *Empetrum* in degraded tundra ecosystems, like those in Iceland, may vary between populations. We asked the following research questions: Q1) Do the allelopathic capacities differ between the *hermaphroditum* and *nigrum* groups? Q2) Do the allelopathic capacities differ between male and female plants within the *nigrum* group? Q3) Do foliar BIII and DDB concentrations differ between *Empetrum* intra-specific groups and sexes? We hypothesized that: H1) the *hermaphroditum* group has stronger allelopathic capacities than the *nigrum* group, H2) males have stronger allelopathic capacities than females of the *nigrum* group, and H3) the *hermaphroditum* group has higher BIII concentrations than the *nigrum* group, and males have higher DDB concentrations than females of the *nigrum* group. To test H1 and H2, we ran a series of bioassays assessing the inhibition of *Festuca richardsonii* seed germination and seedling root elongation by leaves from the different *Empetrum* intra-specific groups and sexes. *Festuca* was chosen as the target species because it is a palatable and fast-growing grass species native to Iceland. To test H3, we quantified BIII and DDB concentrations of the *Empetrum* leaves used for the bioassays, using a targeted liquid chromatography-mass spectrometry (LC-MS) approach. To obtain more accurate quantifications, we evaluated the sample-matrix effect on CS detection, which can lead to an underestimation or overestimation of the calculated foliar concentrations (Jorge et al. 2016).

Material and methods

Empetrum leaf sampling

We collected *Empetrum* leaves on 6 July 2023 in a dry grass-land located close to Reykjavik (64°12'N, 21°38'W, 88 m a.s.l.). This site was chosen because there the *Empetrum* individuals are easily distinguishable because they grow a few meters apart and can thereby readily be identified into the different *Empetrum* intra-specific groups and sexes.

Five to seven branches were cut from five different individuals of each *Empetrum* intra-specific group and sex (i.e. *hermaphroditum*, male *nigrum*, and female *nigrum* plants). We identified *Empetrum* individuals as *hermaphroditum* if both berries and stamens were found on the same branches, and as *nigrum* male or female if either only stamens or berries were found on the same branches, respectively (Fig. 1A). The branches were immediately put in tea filters (130 × 100 mm, 60/420.21.04, Finum), inside Ziploc bags containing

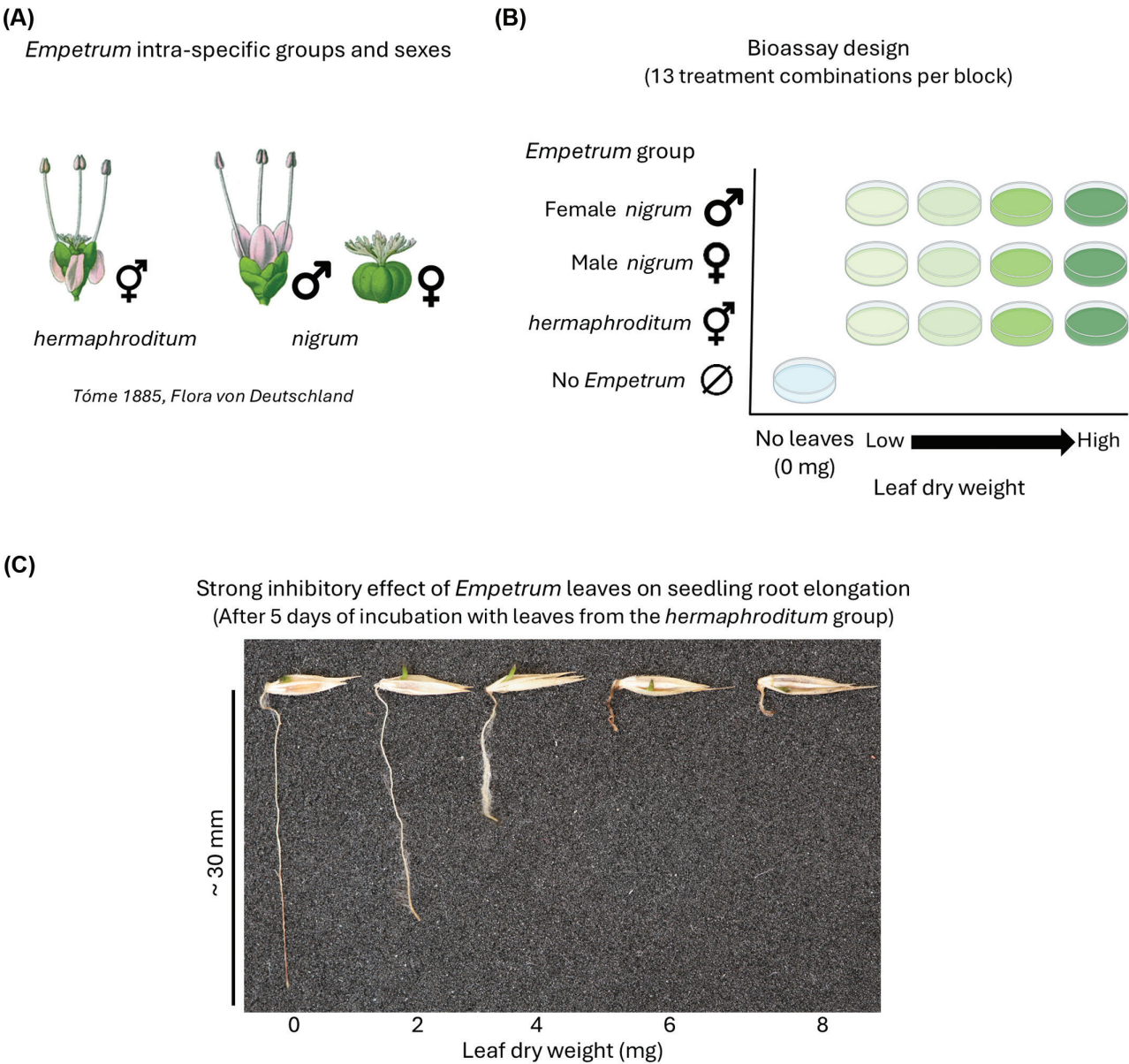


Figure 1. Experimental design. (A) Leaf-material was collected from different *Empetrum* intra-specific groups and sexes: distinguished by their synoecious flowers (i.e. hermaphrodite individuals; the *hermaphroditum* group) and dioecious flowers (i.e. male and female individuals; the *nigrum* group). (B) Bioassays were established in a factorial block design with 13 treatment combinations of the factors ‘*Empetrum* group’ and ‘Leaf dry weight’ in each block. The level ‘No *Empetrum*’ of the factor ‘*Empetrum* group’ confounded with the level ‘0 mg’ of the factor ‘Leaf dry weight’. Illustration created partly by using BioRender.com. (C) Strong inhibitory effects of *Empetrum* leaves were observed on seedling root elongation. Photograph of seedlings with a gradient of leaves (0–8 mg) from the *hermaphroditum* group. All the shoots of the seedlings looked similar and were excised.

silica gel (Granular 0.2–1.0 mm, 10 087, Sigma Aldrich), to reduce metabolic conversions.

In the laboratory, fully developed green leaves were retrieved from the collected branches. A sub-set of leaves from each *Empetrum* individual was homogenized and analysed for BIII and DDB concentrations by LC-MS. The remaining mature leaves were pooled for each intra-specific group and sex for the bioassay assessment of allelopathic capacities. All leaf-material was stored dry, in Ziploc bags with silica beads, until further use.

Bioassay design

We assessed variations in allelopathic capacities between the different *Empetrum* intra-specific groups and sexes, by a series of bioassays modified from González et al. (2015) and Valsdóttir (2021). We constructed the bioassays by first adding *Empetrum* leaves to the bottom of Petri dishes (60 mm, 82.1194.500, Sarstedt AG and Co. KG). Subsequently, we placed two filter papers on top of the leaves (Grade 118, Retention 15 µm, 47 mm, 110.047, Frisette ApS) and then we added 1.5 mL of distilled water. Finally, we placed *Festuca* seeds or seedlings (Provided by the Icelandic Soil Conservation Service, harvested 2021–2022) on top of the filter papers.

In one bioassay (Table 1, Bioassay 1), the allelopathic capacities of the different *Empetrum* intra-specific groups and sexes were assessed as the inhibition of *Festuca* seed germination, i.e. the protrusion of the radicle. Ten seeds were added to each Petri dish and seed germination was measured by recording the number of germinated seeds out of ten. As such, each Petri dish corresponded to one replicate (Table 1, Bioassay 1). This bioassay was established in a factorial block design with ten blocks. Each block contained Petri dishes for each combination of the two factors: '*Empetrum* group' (Levels: No *Empetrum*; *hermaphroditum*, male *nigrum*, and female *nigrum*) and 'Leaf dry weight' (Levels: 0, 10, 20, 40, and 80 mg). These levels of leaf dry weight were used for comparable reasons with previous studies, such as González et al. (2015) and Valsdóttir (2021). The leaf dry weight level of '0 mg' confounded with the *Empetrum* group level of 'No *Empetrum*', and contained only water, filter papers, and *Festuca* seeds. As such, each block included 13 (i.e. $3 \times 4 + 1$) different combinations (Fig. 1B; Table 1). Germination was recorded 10 times during 31 days of incubation, i.e. approximately every third day (Table 1, Bioassay 1).

In another bioassay (Table 1, Bioassay 2), seedling root elongation was assessed by measuring the total root length of individual seedlings after seven days of incubation. Five *Festuca* seedlings (i.e. pre-germinated seeds with 2 mm of root) were added to each Petri dish, and each seedling was considered as one replicate (Table 1, Bioassay 2). This bioassay was established in a factorial block design with three blocks. Each block contained 13 Petri dishes, one for each combination of the two factors: '*Empetrum* group' and 'Leaf dry weight', as described above (Fig. 1B). Because of a strong inhibitory effect of *Empetrum* leaves observed on *Festuca* root elongation (Fig. 1C), a third bioassay was done (Table 1, Bioassay 3). The third bioassay was similar to the one described above, except that it was established with lower levels of the factor 'Leaf dry weight' (Levels: 0, 2, 4, 6, and 8 mg; Table 1, Bioassay 3). This bioassay was incubated for nine days (Table 1, Bioassay 3).

In all bioassays, all Petri dishes were sealed with parafilm to avoid water-loss and incubated in growth chambers at constant light and 20°C. During incubation, the Petri dishes were regularly moved to a new location within the growth chamber to account for any edge-effects.

Chemical analysis and data processing

For each sample, methanol boiling extraction (De Vos et al. 2007) was performed on approximately 25 mg of homogenized leaf tissue in 500 µL of extraction buffer (85% methanol, 0.1% formic acid, and 250 µM caffeine as internal standard), as in Ryde et al. (2021). All extractions were diluted 20 times and filtered through a membrane filter (0.22 µm, Merck Millipore) before chemical analysis by LC-MS.

Chromatographic separation was done using a Dionex Ultimate 3000RS system (Thermo Fisher Scientific). Two µL of each sample was separated in a reverse-phase Phenomenex Kinetex column (100 Å, 1.7 µm C18, 150 x 2.1 mm, Phenomenex Inc.) held at a constant flow rate of 0.3 mL min⁻¹, cooling autosampler temperature of 10°C, and column oven temperature of 40°C. The gradient elution was as follows: initially 98% solvent A (i.e. 0.05% formic acid in water; Pierce LC-MS grade formic acid, Thermo Fisher Scientific) and 2% solvent B (i.e. 0.05% formic acid in acetonitrile; HPLC-grade, ≥ 99.9% purity, Sigma Aldrich) were held for 1 minute; then, a linear increase of solvent B to 10% in 1 minute, to 40% in 10 minutes, to 60% in 10 minutes, and to 100% in 1 minute, which was then held for 1 minute.

Table 1. Bioassay design. Listed are the different bioassays (1–3), allelopathy assessment (i.e. inhibition of *Festuca* seed germination or seedling root elongation), number of treatment combinations, blocks, seeds/seedlings per Petri dish, replicates, recordings, observations, and incubation days for each bioassay.

Bioassay	Assessment	Treatment combinations (Petri dishes per block)	Blocks	Seeds/seedlings per Petri dish	Replicates	Recordings	Total number of observations	Incubation days
1	Seed germination	13	10	10	Petri dishes (1 per block), 10 x 1: n=10	10 times during incubation	13 x 10 x 1 x 10: N=1300	31
2	Seedling root elongation		3	5	Seedlings (5 per block), 3 x 5: n=15	1 time after end incubation	13 x 3 x 5 x 1: N=195	7
3								9

Finally, solvent B was returned to 2% and held for 5 minutes to re-equilibrate the column. Mass detection was done on a Compact quadrupole time-of-flight mass spectrometer, with an electrospray ionization source (Bruker Daltonics), as in Dos Santos et al. (2019), detecting compounds with 50–1400 mass-to-charge (m/z) ratios in positive mode (see detailed information of mass spectrometer parameter settings in the Supporting information). To establish standard curves for compound quantification, authentic standards (from Alb Technology Limited) of BIII ($\geq 95\%$ purity) and DDB ($\geq 98\%$ purity) were run alongside the samples. In addition, to monitor potential contamination, extraction blanks and 100% methanol blanks were run throughout the batch of samples.

Raw data was calibrated (see parameters in the Supporting information) in the Bruker compass data analysis ver. 4.3 software (Bruker Daltonik GmbH 2014). Data processing was then done in the MZmine ver. 4.5 software by mzio GmbH (Schmid et al. 2023) in centroid mode with a signal-noise level of 400, and an m/z and retention time tolerance of 0.005 and 0.02 minutes, respectively (see all parameter settings in the Supporting information). Contamination from the extraction procedure and analytical chemical system were removed by subtracting detected masses from the extraction blanks and 100% methanol blanks.

Compound quantification and sample-matrix effects

To quantify BIII and DDB foliar concentrations, standard curves were run in a 7-point dilution series with linear ranges of 0–150 μM and 0–37.5 μM , respectively. Furthermore, we have previously run a BIII standard curve on an identical instrument, with the exact same chromatographic method

and mass spectrometer settings, that had a linear range of 50–600 μM (Supporting information). To assess the sample-matrix effect on compound signal (CS) detection (i.e. compound peak area), standard curves were created with the compounds spiked in blank samples (100% methanol) and in sample-matrix (i.e. methanol extractions of *Empetrum* leaves as described above). The sample-matrix used, was taken from a leaf-sample of the *nigrum* group, of which preliminary results had showed negligible BIII and DDB compound signals. The matrix effect (ME) was then calculated in accordance with Jorge et al. (2016):

$$\text{ME} (\%) = \frac{\text{CS}_{\text{spiked matrix}} - \text{CS}_{\text{non-spiked matrix}}}{\text{CS}_{\text{spiked blank sample}}} \times 100$$

ME = 100% indicates no matrix effect, while ME < 100% indicates ion suppression and ME > 100% indicates ion enhancement, i.e. an underestimation and overestimation of compound quantities, respectively (Jorge et al. 2016).

We calculated an ME of approximately 82–91% for BIII concentrations above 19 μM (Fig. 2A), and of 87–95% for DDB concentrations above 9 μM (Fig. 2B). As such, BIII and DDB absolute quantities calculation based on the standard curves created without sample-matrix in our study, could result in an up to 13–18% underestimation at higher compound concentrations. Therefore, we aimed to quantify BIII and DDB concentrations (mg compound g^{-1} dry weight leaf-sample) by using the linear relationship of standard curves run in sample-matrix (Fig. 2), along with the

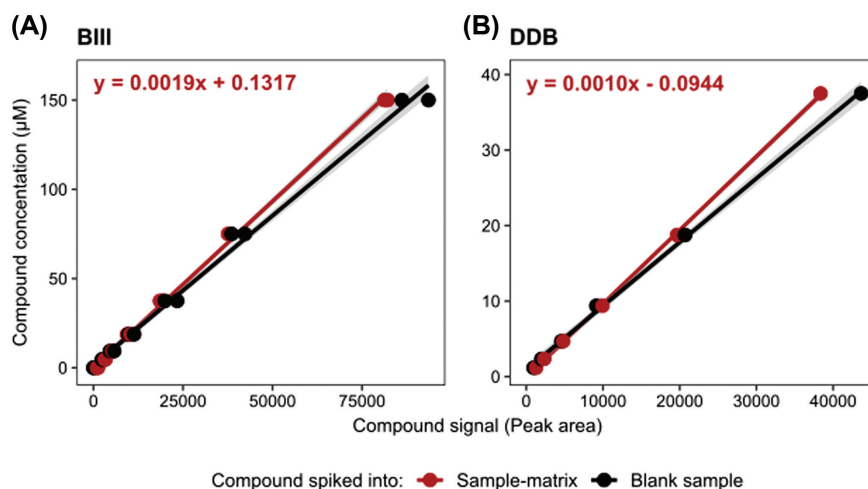


Figure 2. Standard curves of BIII and DDB spiked in sample-matrix and blank-samples. Linear regression lines with known compound concentrations (μM) as a function of detected compound signals (peak area), from a 7-point dilution series of the compounds BIII (A) and DDB (B). The BIII dilution series ($n=2$) consisted of the concentrations 0.0, 4.7, 9.4, 18.8, 37.5, 75, and 150 μM , while the DDB dilution series ($n=1$) consisted of the concentrations 0.0, 1.2, 2.3, 4.7, 9.4, 18.8, and 37.5 μM . The grey area shows the 95% confidence interval of the regression lines for BIII and DDB spiked in sample-matrix (red) and in blank-sample (black). The first point in the DDB dilution series (i.e. 0.0 μM), for the standard curve spiked in sample-matrix and blank samples, had a detected compound signal (CS) of nearly 0 and was removed from the figure (B) to enable the calculation of the 95% confidence interval. A linear regression equation is shown for the standard curves with compounds spiked in sample-matrix.

extraction volume, dilution factor, instrument injection volume, and sample dry weight.

Statistics

All statistical analyses were done in the R statistical software ver. 4.3.3 (www.r-project.org), where we established mixed-effects models using the 'lme4' package (Bates et al. 2015).

To analyse variations in seed germination inhibition, a model was established with the response variable 'germination', the explanatory variables 'Empetrum group', 'leaf-density', and 'incubation day' (Levels: 1–10), and the random factor 'block'. The response variable 'germination' was binomially distributed and therefore analysed by a generalized linear mixed-effects model using the *glmer* function.

To analyse variations in root elongation inhibition, two models (one for each root elongation bioassay) were established with the response variable 'root elongation', the explanatory variables 'Empetrum group' and 'leaf-density', and the random factors 'block' and 'Petri dish'. The first model analysed the series of higher leaf-density levels (0 mg and 10–80 mg), and the second model analysed the lower leaf-density levels (0 mg and 2–8 mg). The response variable 'root elongation' was analysed by linear mixed-effects models using the *lmer* function. To fulfil the requirements of normal distribution and homogeneity of variance, 'root elongation' was log-transformed in the first model (with higher leaf-density levels) and square root-transformed in the second model (with lower leaf-density levels).

To analyse variations in compound quantities of the leaf-material used for the bioassays, all compound concentrations were first normalized, i.e. divided by the detected peak area of the internal standard caffeine. To analyse differences between intra-specific groups and sexes, a linear model, using the *lm* function, was established with the response variable 'compound concentration', the explanatory variable 'Empetrum group', and no random factor. The response variable was log-transformed to fulfil the requirements of normal distribution and homogeneity of variance.

Full and reduced models were compared by ANOVA (*anova* function) to analyse the overall statistical effects of all models. In models analysing seed germination and root elongation, pairwise comparisons were made between the *Empetrum* groups for each leaf-density level, and between the leaf-density level '0 mg' and the other leaf-density levels for each *Empetrum* group, whereas for the model analysing compound concentrations, pairwise comparisons were just made between *Empetrum* groups (Supporting information). Furthermore, for the model analysing seed germination inhibition, pairwise comparisons were made between *Empetrum* groups for each incubation day, and between incubation days for each *Empetrum* group and leaf-density level (Supporting information). All pairwise comparisons were done by using the *emmeans* function from the 'emmeans' package (Lenth 2023). Bonferroni corrections were applied for models including the response variables 'germination', while Tukey's corrections were applied for models including the response variable 'root elongation' or 'compound concentration'.

Results

Differences in allelopathic capacities between *Empetrum* intra-specific groups (Q1)

Festuca seed germination was inhibited more strongly by the *hermaphroditum* group than the *nigrum* group (Fig. 3). After 31 days of incubation, 40 and 80 mg of *hermaphroditum* leaves inhibited germination by approximately 11% ($p=0.03$; Supporting information) and 47% ($p < 0.001$; Supporting information), respectively, more than *nigrum* leaves (average of male and female individuals; Fig. 3A). Compared to the germination of seeds incubated with no *Empetrum* leaves, leaf-densities of 40 and 80 mg inhibited germination by approximately 14–25% ($p < 0.05$; Supporting information) and 33–64% ($p < 0.001$; Supporting information), respectively, depending on the intra-specific group (Fig. 3A). Low *Empetrum* leaf-densities (10 and 20 mg) did not inhibit seed germination after 31 days of incubation (Fig. 3A; $p > 0.1$; Supporting information). However, compared to seeds incubated with no *Empetrum*, germination of seeds with 20 mg of *hermaphroditum* leaves was delayed during the first 21 days of incubation ($p < 0.05$; Supporting information), whereas the germination of seeds incubated with *nigrum* leaves were delayed only during the first nine days ($p < 0.05$; Supporting information).

There were no major differences in the inhibition of *Festuca* root elongation between the *Empetrum* intra-specific groups at high (Fig. 3B) and low (Fig. 3C) levels of leaf dry weight. Nevertheless, the *hermaphroditum* group did inhibit root elongation by approximately 24% more than males of the *nigrum* group at a leaf dry weight of 4 mg ($p < 0.01$; Supporting information). Furthermore, although there were no significant differences overall between the intra-specific groups when tested against each other ($p > 0.099$; Supporting information), 2 mg (equivalent to a single leaf) of leaves from the *hermaphroditum* group inhibited root elongation by 30% (Fig. 3C; $p=0.058$; Supporting information), whereas 2 mg of leaves from the *nigrum* group did not (Fig. 3C; $p > 0.9$; Supporting information). Compared to the root elongation of seedlings incubated with no *Empetrum* leaves, *Festuca* root growth was strongly inhibited by very low *Empetrum* leaf dry weight levels (Fig. 3C). A leaf dry weight level of 4–8 mg reduced total root length by approximately 52–75% on average (Fig. 3C; $p < 0.01$; Supporting information), and a 10–80 mg leaf dry weight reduced total root length by approximately 80–94% (Fig. 3B; $p < 0.001$; Supporting information).

Differences in allelopathic capacities between sexes of the *nigrum* group (Q2)

Festuca seed germination did not differ between sexes of the *nigrum* group after 31 days of incubation (Fig. 4; $p > 0.2$; Supporting information) but was delayed more by *nigrum* males than females during the incubation period (Fig. 4; Supporting information). Despite no significant difference between sexes after 31 days of incubation, germination was significantly inhibited by 40 mg of leaves from *nigrum* males (Fig. 4; $p=0.042$; Supporting information) but not

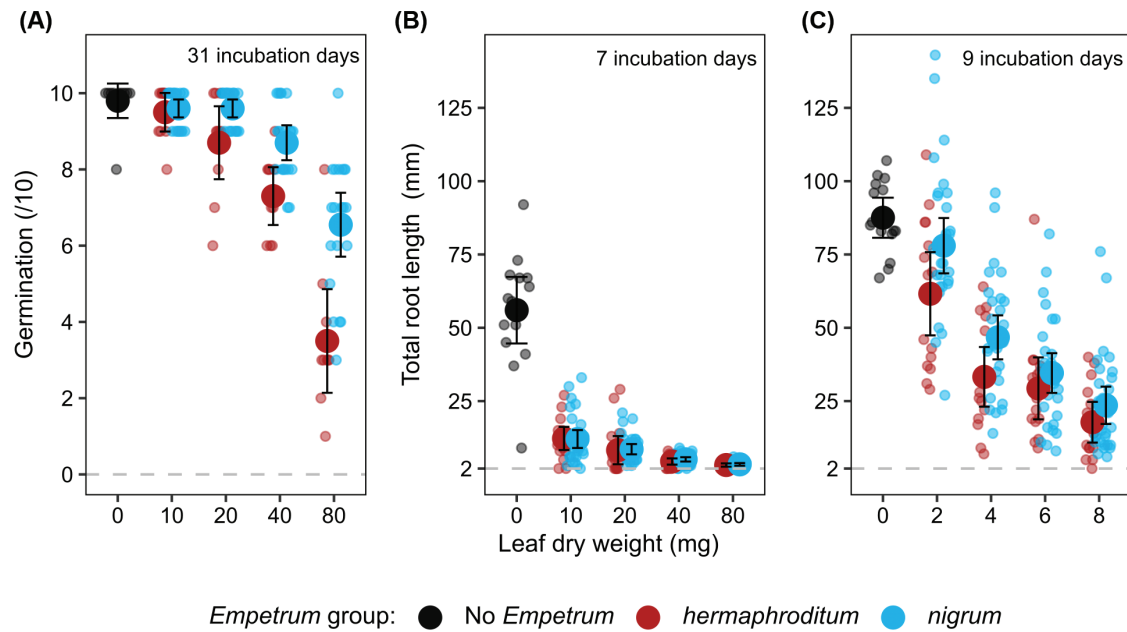


Figure 3. Differences in seed germination and seedling root elongation inhibition between *Empetrum* groups. *Festuca* seed germination (A; $n = 10$) and seedling root elongation ($n = 15$) with high (B) and low (C; note the different x-axis scales) levels of *Empetrum* leaves from the intra-specific groups *hermaphroditum* and *nigrum* (averaged across male and female individuals), and no *Empetrum* (0 mg), shown as raw values (small dots) and predicted means (big dots) $\pm$ 95% confidence intervals. Dashed line indicates a seed germination of 0% (A) and the initial root length of seedlings, i.e. 2 mm (B–C).

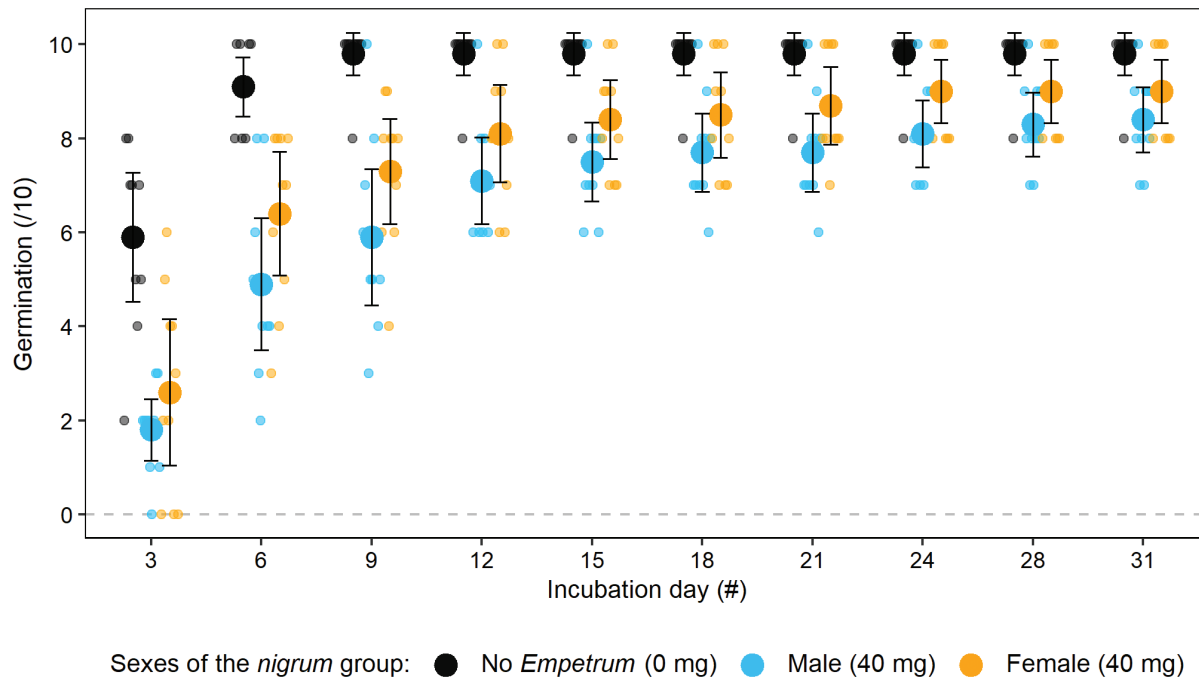


Figure 4. Differences in seed germination inhibition between sexes of the *nigrum* group. *Festuca* seed germination ($n = 10$) with no *Empetrum* (0 mg) and *Empetrum* leaves (40 mg) from the male and female sexes of the *nigrum* group, shown as raw values (small dots) and predicted means (big dots) $\pm$ 95% confidence intervals. Dashed line indicates a seed germination of 0%.

from *nigrum* females (Fig. 4; $p=0.4$; Supporting information), compared to seeds incubated with no *Empetrum*. At a leaf dry weight of 40 mg, *nigrum* females only delayed seed germination during the first 15 days of incubation (Fig. 4; $p < 0.05$; Supporting information). At lower leaf dry weight levels (10–20 mg), *nigrum* males delayed seed germination, compared to seeds incubated with no *Empetrum*, during the first nine days of incubation ($p < 0.05$; Supporting information). In comparison, *nigrum* females did not delay seed germination at a leaf dry weight of 10 mg and only delayed seed germination during the first three days at a leaf dry weight of 20 mg ($p < 0.05$; Supporting information). *Festuca* seedling root elongation was not affected differently by sexes of the *nigrum* group (Supporting information).

Differences in bibenzyl concentration between *Empetrum* intra-specific groups and sexes (Q3)

Targeted LC-MS analysis detected BIII in samples from all *Empetrum* intra-specific groups and sexes (Fig. 5), whereas DDB was not detected in any samples. The *hermaphroditum* group varied in BIII concentrations from 0.01 to 44.4 mg g⁻¹ leaf dry weight with a mean ($\pm$ SE) of 24.2 ± 6.8 mg g⁻¹ leaf dry weight. In comparison, BIII concentrations were significantly lower in males and females of the *nigrum* group ($p=0.019$; Supporting information), ranging from 0.01–0.43 mg g⁻¹ leaf dry weight, with an overall mean ($\pm$ SE) of 0.3 ± 0.2 mg g⁻¹ leaf dry weight (Fig. 5). No difference in BIII concentrations between male and female individuals of the *nigrum* group was observed (Fig. 5; $p=0.8$; Supporting information).

Discussion

Here we measured the allelopathic capacity of different intra-specific groups and sexes of Icelandic *Empetrum*

populations. We report a stronger allelopathic capacity of the *hermaphroditum* group compared to the *nigrum* group (H1), in accordance with Nilsson et al. (2000) who studied a Scandinavian *Empetrum* population. Stronger allelopathic capacity of males compared to females of the *nigrum* group was also observed (H2), corresponding with a previous sex-related allelopathy study on the invasive Peruvian peppertree *S. molle* (Avendaño-González et al. 2016). In addition, a higher concentration of the allelopathic bibenzyl BIII was measured in leaves from the *hermaphroditum* group compared to leaves from the *nigrum* group, which may partly explain their differences in allelopathic capacity (H3). Differences in allelopathic capacity between intra-specific groups and sexes, however, depended on the developmental stage of the tested grass species *Festuca*, i.e. the susceptibility of seed germination and seedling root elongation to *Empetrum* allelochemicals. This might suggest that differences in allelopathic capacities between *Empetrum* intra-specific groups and sexes are more complicated than first anticipated. Finally, we saw *Festuca* inhibition by *Empetrum* leaf dry weight levels on seed germination and root elongation as low as 40 and 2 mg (equivalent to a single leaf in Petri dishes of 28.3 cm²), respectively, which emphasizes the strong allelopathic capacities of *Empetrum*. BIII concentrations detected in leaves from the *hermaphroditum* group, were overall in the same range as previously reported levels measured in a Scandinavian *Empetrum* (*hermaphroditum*) population (González et al. 2015). The measured maximum BIII concentration, however, was considerably higher in our study compared to González et al. (2015), and may be a result of population-specific variation in BIII production. This could also be a result of the suppression of CS detection imposed by the sample-matrix effect reported in our study, indicating that previous quantification of *Empetrum* foliar BIII concentrations may have been underestimated.

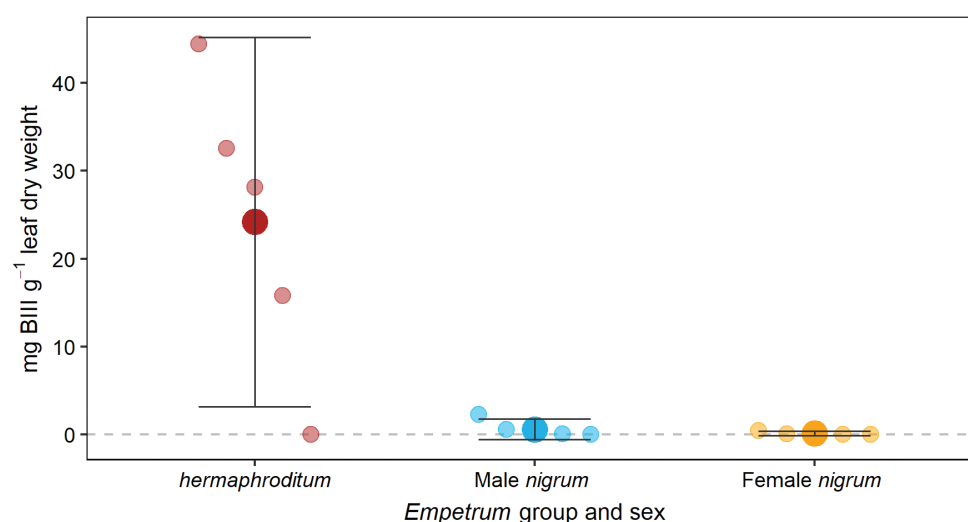


Figure 5. Differences in foliar BIII concentrations between *Empetrum* groups and sexes. Raw data points (small dots) shown with means (large dots) $\pm$ 95% confidence limits of leaves from the *Empetrum* intra-specific groups and sexes, i.e. *hermaphroditum*, male *nigrum*, and female *nigrum* ($n=5$). The grey dashed line indicates a compound concentration of 0 mg.

We found intra-specific differences in allelopathic effects on seed germination but not seedling root elongation of *Festuca*. Nilsson et al. (2000) reported that leaf-extracts from the *hermaphroditum* group inhibited *Pinus sylvestris* and *Populus tremulus* seed germination and root elongation more than the *nigrum* group in laboratory bioassays. These results were related to foliar BIII concentrations of the *hermaphroditum* group (i.e. 3.2% leaf dry weight; Wallstedt et al. 1997) being higher than foliar DDB concentrations of the *nigrum* group (i.e. 0.9% leaf dry weight; Jarevång et al. 1998), and that BIII is more phytotoxic than DDB (Nilsson et al. 2000). Similarly, we observed that the *hermaphroditum* group had much higher quantities of BIII and inhibited *Festuca* seed germination more than the *nigrum* group. Interestingly, DDB was not detected in the measured Icelandic *Empetrum* plants, which may suggest chemical diversity between Icelandic and Scandinavian *Empetrum* populations, but a wider population analysis is required to establish broad chemical profiles.

In our study, *Festuca* seedling root elongation was generally impacted much more strongly than seed germination with no noteworthy differences between *Empetrum* intra-specific groups. Valsdóttir (2021), observed stronger *Festuca* seed germination inhibition by a pooled leaf-sample from an *Empetrum* population consisting only of *hermaphroditum* plants (i.e. 100% abundance), compared to an *Empetrum* population consisting nearly only of *nigrum* plants (i.e. 91% abundance). Furthermore, Valsdóttir (2021) observed no inhibition differences in *Festuca* root elongation between the two populations. This corresponds well with our results, supporting that the observed differences between populations made by Valsdóttir (2021), are due to differences in allelopathic capacities related to allelochemical variation between *Empetrum* intra-specific groups. Nevertheless, the discrepancies regarding root elongation inhibition between our study and that of Nilsson et al. (2000), might be due to variation in the susceptibility to *Empetrum* allelochemicals between plant species and developmental stages (González et al. 2015). As such, the importance of proportional abundances of intra-specific groups within *Empetrum* populations, likely depends on the susceptibility of various ecologically important species.

We observed differences in allelopathic capacities between sexes of the *nigrum* group, i.e. males delayed *Festuca* seed germination more than females. One of the consequences of delayed seed germination can be reduced plant fitness, such as seedling survival (Akiyama and Ågren 2014) and decreased competitive ability (Blackford et al. 2020). Accordingly, our results suggest a stronger allelopathic capacity of male individuals. In contrast to differences observed between the intra-specific groups (*hermaphroditum* and *nigrum*), no significant difference in BIII levels was detected between sexes. In general, the levels of BIII were low in both male and female plants, which aligns with previous reports (Jårevång et al. 1998) and suggests that other allelochemicals play a role in determining the different allelopathic capacities between sexes of the *nigrum* group. This is supported by Oden et al (1992), who estimated that BIII only accounted for approximately 28% of the allelopathic capacity of *Empetrum*, and González et al.

(2015), who observed no correlation between BIII foliar concentrations and *Empetrum* allelopathic capacities.

In a similar study, Avendaño-González et al. (2016) showed that male *S. molle* individuals had greater allelopathic capacities than females and speculate that allelochemical variation is an important determining factor. These two systems provide a unique opportunity for further untargeted metabolomic analysis to determine key metabolites that may drive negative plant-plant interactions caused by allelopathy. Based on the outcomes of the present study and Avendaño-González et al. (2016), it is speculated that female plants may invest less resources in allelochemicals than males, due to a greater allocation towards reproductive tissue (Ågren et al. 1999). Differential allocation of resources between male and female plants has previously been shown in the context of herbivory, where the opposite effect has been reported, i.e. females are less palatable than male individuals, with a greater defence compound accumulation (Cornelissen and Stiling 2005). These contrasting patterns highlight the highly dynamic and multi-functional role of plant specialized metabolites. Given that studies measuring chemical defence mechanisms between male and female plants are restricted to relatively few taxa, overall differences between male and female resource allocation may not be as significant as originally hypothesized (Avila-Sakar and Romanow 2012). For example, in some cases males may also be genetically disposed to produce specific allelochemicals with a higher degree of bioactivity (Muzell Trezzi et al. 2016). Furthermore, environmental stressors can affect sexual differences in reproductive allocation and investment in other plant functions, depending on species and stress types (Liu et al. 2021).

We conclude that the investigated *Empetrum* intra-specific groups and sexes (*hermaphroditum*, male *nigrum*, and female *nigrum*) affect *Festuca* differently depending on developmental stage (seed germination and seedling root elongation). The observed differences are likely due to quantitative and qualitative allelochemical variation resulting in varying allelopathic capacities between *Empetrum* intra-specific groups and sexes, and variation in the susceptibility to *Empetrum* allelochemicals between plant developmental stages. Allelochemicals, such as BIII, could partly explain these differences. Our results suggest that the role of *Empetrum* in degraded tundra ecosystems, like those in Iceland, depends on the relative abundance of different *Empetrum* intra-specific groups and sexes. To further our understanding of the role of *Empetrum* in degraded tundra ecosystems, the sensitivity of several ecologically important species should be investigated. In addition, more detailed chemical analyses and bioassays are required to uncover the allelochemicals responsible for the allelopathic capacity of *Empetrum*. The allelopathic differences between intra-specific groups and sexes observed in our study, offer a unique biological system that can be utilized to identify more bioactive compounds of *Empetrum*.

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Author contributions

Ingvild Ryde: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Investigation (lead); Methodology (lead); Project administration (equal); Supervision (supporting); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Marika Halmová:** Investigation (supporting); Methodology (supporting). **Kari Anne Bråthen:** Conceptualization (equal); Methodology (supporting); Supervision (equal); Validation (equal); Writing – review and editing (equal). **Elizabeth H. J. Neilson:** Conceptualization (equal); Methodology (supporting); Validation (equal); Writing – review and editing (equal). **Ingibjörg S. Jónsdóttir:** Conceptualization (equal); Funding acquisition (lead); Methodology (supporting); Project administration (equal); Resources (lead); Supervision (lead); Validation (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.73n5tb38z> (Ryde et al. 2025b).

Supporting information

The Supporting information associated with this article is available with the online version.

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