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Batatasin-III and the allelopathic capacity of *Empetrum nigrum*

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Batatasin-III (3,3-dihydroxy-5-methoxybibenzyl) is a phenolic compound associated with the allelopathic effect of the evergreen dwarf shrub *Empetrum nigrum*, and has been referred to as the causal factor for the species being successful in dominating extensive ecosystems. Yet, only a few plant species have been tested for their response to batatasin-III, and little is known about whether environmental factors modify this allelopathic effect. In this study, we tested the inhibitory effect of purified batatasin-III through bioassays on 24 vascular plant species and, for certain species, we tested if this effect depended on growth substrate (mineral vs organic substrate), pH, and fertilization. Moreover, we tested if batatasin-III predicted the allelopathic effect of *E. nigrum* by analyzing the inhibitory effect of *E. nigrum* leaves and humus in relation to their batatasin-III content. Our results confirmed batatasin-III as a stable compound capable of inhibiting germination and/or mean root elongation in all of the tested species, but this effect was modified by growth substrate. Surprisingly, the measured batatasin-III content of *E. nigrum* leaves (mean value 19.7 ± 10.8 (SE) mg g^{-1}) and humus (mean value of 1 ± 1.5 (SE) μg g^{-1}) did not predict the inhibitory effect on mean root elongation. Although batatasin-III was found to be phytotoxic to all the tested species, we conclude that this substance alone should not be used as a proxy for the allelopathic effect of *E. nigrum*.

Allelopathy, i.e. the chemical interference of one plant on growth and distribution of other plants (Rice 1984), is highly influential to how communities are organized. Chemical interference is often recognized by the phytotoxic activity of identified chemical compounds (phytochemicals), of which phenolics represent a major group (Harborne 1993). Phytochemicals are usually secondary metabolites introduced to the environment through foliar leaching, root exudates, volatilization or litter decomposition. Factors playing an important role in the toxicity of phytochemicals include their concentration, residence time and fate in the environment (Fisher 1987, Horner et al. 1988).

Batatasin-III (3,3′-dihydroxy-5-methoxybibenzyl) is a phenolic compound of class dihydrostilbene recognized for its chemical interference with plant growth (Saito et al. 1975). Batatasin-III was first isolated and identified from dormant bulbils of *Dioscorea batatas* where it was suggested to be involved in the control of bulb dormancy (Hashimoto et al. 1972, Hasegawa and Hashimoto 1975). Furthermore, batatasin-III has been shown to inhibit seed germination of *Populus tremula* (Odén et al. 1992, Zackrisson and Nilsson 1992), root elongation in *Pinus sylvestris* (Nilsson and Zackrisson 1992, Nilsson et al. 1993), and both photosynthesis and ammonium uptake in birch (*Betula pendula*) seedlings (Wallstedt et al. 2001a, 2001b). Hence, there are multiple ways by which batatasin-III can have chemical interference with plants, yet only a handful species have been tested.

The ericaceous species *Empetrum nigrum* (including *E. hermaphroditum*) has been shown to produce batatasin-III, which is released from its green leaves and litter by rain and snowmelt (Odén et al. 1992, Wallstedt et al. 1997, Nilsson et al. 1998). *Empetrum nigrum* is an evergreen dwarf shrub that grows in alpine and circumpolar areas where it forms dense mats of nearly monospecific vegetation (Bell and Tallis 1973). It grows slowly, dominates where disturbance is infrequent (Tybirk et al. 2000) and herbivory is low, mainly feeding on the berries (Brathen et al. 2007). The dominance of *E. nigrum* in forest understory vegetation has been a major concern in the recent decade (Tybirk et al. 2000), mainly due to its negative effect on reforestation (Nilsson and Zackrisson 1992, Zackrisson and Nilsson 1992, Nilsson and Wardle 2005) and commercial forest productivity (Pellissier and Souto 1999). The production of batatasin-III has been referred to as the causal factor for *E. nigrum* being successful in dominating extensive ecosystems (Nilsson and Wardle 2005, Brathen et al. 2010) due to negative effects on germination of other plant species, soil microbial activity, and plant litter decomposition rates (Jonasson 1983, Wardle and Lavelle 1997). Therefore, ecological

research has focused on batatasin-III as the main metabolite responsible for the chemical interference exerted by *E. nigrum*. The concentration of batatasin-III in *E. nigrum* leaves can be 10-fold higher than other phenolic compounds and may constitute up to 6% of the leaf dry weight of the first and second year shoots (Gallet et al. 1999). However, how well batatasin-III concentration in leaves of *E. nigrum* predicts its level of phytotoxic activity has only been tested on the tree species *P. tremula* (Nilsson et al. 1998), where it retarded seed germination. Furthermore, batatasin-III appears stable in soil environment where it can accumulate to sufficient levels as to influence biological activity (Wallstedt et al. 1997, Gallet et al. 1999, Brannas et al. 2004, Brathen et al. 2010), but little is known about how soil properties such as pH, fertilization or substrate type directly influence the phytotoxicity of batatasin-III.

The purpose of the present study was to improve our understanding of the chemical interference of *E. nigrum* by focusing on the role of batatasin-III as a growth inhibiting substance. Through bioassays we quantified the variation in inhibitory growth activity of purified batatasin III in 24 vascular plant species. Second, for a range of these species, we tested the effect of different growth substrates, pH and fertilization on the inhibitory growth activity of purified batatasin-III. We expected to find a stronger effect of batatasin-III in substrates with less organic matter (Wallstedt et al. 2005), lower pH (Brannas et al. 2004) and no fertilization (Nilsson et al. 2002, Shevtsova et al. 2005). And finally, we studied the inhibitory growth effect of *E. nigrum* leaves and humus, where we expected to find a positive relationship between the measured content of batatasin-III and its inhibitory effect.

Material and methods

Empetrum nigrum is an evergreen dwarf shrub. The circumpolar race of this species was previously known as *E. hermaphroditum* or *E. nigrum* subsp. *hermaphroditum* while *E. nigrum* subsp. *nigrum* was believed to have a more southernly boreal distribution, but according to the Pan Arctic Flora (<http://nhm2.uio.no/paf>) these two taxa are no longer recognized. However, all references used in the present study refer to the northern race previously known as *E. hermaphroditum* unless otherwise stated.

We carried out three bioassays testing the inhibitory growth effect of purified batatasin-III, and one bioassay testing the effect of leaves and humus of *E. nigrum* on seed germination and mean root elongation of multiple species. For all bioassays we used petri dishes (4.5 cm in diameter) with two Whatmann no. 1 filter papers covered with lids and plastic film in order to avoid water loss, with a temperature of 20°C and 24-h simulated daylight (Philips TLD 489 fluorescent tubes), except bioassay 1 for root elongation that was performed in darkness. For germination tests, between 20 and 100 seeds were placed in each petri dish depending on the availability of seeds for each species, while for root elongation tests, ten pre-germinated seeds with a radicle of 1 mm were placed in each petri dish. Stock solutions of batatasin-III for bioassays 1–3 were prepared by dissolving batatasin-III powder in methanol. The remaining methods were specific to each bioassay and are presented below.

Bioassay 1

In bioassay 1, we estimated the inhibitory growth effect of purified batatasin-III, through seed germination and mean root elongation on a total of 24 vascular plant species representing a range of growth forms according to Grime (1979) (Table 1). Seeds of *Arabidopsis* were produced at the phytotron at Univ. of Tromsø. Seeds of *Pinus* and *Picea* were collected from Skogfrøverket (Hamar, Norway) and seeds of the grass species were obtained from Graminor (Bodø, Norway). Seeds of the other species were collected around Troms County. Because seeds of some dwarf shrubs are difficult to germinate under normal growing conditions, seeds of *Calluna vulgaris*, *Vaccinium myrtillus* and *V. vitis-ideae* were stratified before the bioassays started.

Before seeds (to study batatasin-III effect on germination) or pre-germinated seeds (to study batatasin-III effect on root elongation) were added to the petri dishes, doses of 100 µl of the stock solutions were applied to filter paper to give concentrations of 0 (control), 0.1, 0.5, 1, 2 and 4 mM. After adding stock solutions to petri dishes, methanol was allowed to evaporate at room temperature before the bioassays were initiated and 0.5 ml of distilled water was added to moisten the filter paper before placing the seeds or pre-germinated seedlings.

Table 1. List of studied species and concentration range of batatasin-III (mM) giving 50% inhibition of germination or mean root elongation.

Growth form	Species	Germination	Root elongation
Forbs	*Arabidopsis thaliana*	0.0–0.5	–
	Arabis alpina	1–2	0.0–0.5
	Dianthus deltoides	0.5–1.0	–
	Gnaphalium norwegicum	2–4	1–2
	Lactuca sativa	0.5–1.0	1–2
	Saussurea alpina	x	0.5–1.0
	Silene dioica	2–4	0.5–1.0
	Silene uniflora	2–4	0.5–1.0
	Silene deltoides	0.5–1.0	–
	Solidago virgaurea	x	2–4
Grasses	*Agrostis tenuis*	0.0–0.5	0.0–0.5
	Festuca frigida	1–2	0.5–1.0
	Festuca rubra	1–2	1–2
	Phleum pratense	0.0–0.5	0.5–1.0
	Poa pratensis	1–2	1–2
Dwarf shrubs	*Betula nana*	0.0–0.5	0.0–0.5
	Calluna vulgaris	0.0–0.5	0.0–0.5
	Salix pentandra	1–2	–
	Vaccinium myrtillus	2–4	1–2
	Vaccinium vitis-idaea	1–2	–
Trees	*Betula pendula*	–	0.0–0.5
	Betula pubescens	1–2	0.5–1.0
	Picea abies	x	1–2
	Pinus sylvestris	x	1–2

x = No effect of batatasin–III at the highest concentration used (4 mM)
– = No bioassay was performed.

Germination bioassays terminated when no further germination occurred (about 40 days in total). Visible radicle was used as a criterion for germination. Seeds that did not germinate were counted at the end of the test.

For the root elongation bioassay, root elongation of the pre-germinated seeds was measured after incubation for seven days.

Bioassay 2

In bioassay 2, we used pre-germinated seeds of *Pinus sylvestris* and *Lactuca sativa* to test if the effect of batatasin-III on mean root elongation was dependent on different growth substrates (organic vs mineral substrate). The substrates used in this bioassay include sand, humus and peat and were chosen because of their variation in organic matter content which is assumed to be an important factor controlling the biological effect of batatasin-III (Brathen et al. 2010). *Lactuca sativa* is a species commonly used in bioassay studies, while *P. sylvestris* has been shown to be sensitive to *E. nigrum* leaves (Nilsson and Zackrisson 1992) and should therefore be a good indicator for the effects of substrate on batatasin-III activity.

For the bioassays, humus was collected from the top 5 cm layer in meadows with no presence of *E. nigrum* in Ifjordfjellet (Finnmark County, Norway) (300 m a.s.l., 70°44′N, 27°35E), and dried at room temperature for easy transport and storage. It was later filtered through a 2 mm sieve in order to remove debris. The sand and peat used in the bioassays were of commercially available quality.

For the bioassay using *P. sylvestris*, we used as growth substrates 1 g of peat, 1 g sand, or 1 layer of Whatmann no. 1 filter paper as control. Peat and sand were air dried and were moistened with 1.5 ml (sand) or 2.5 ml (peat) of distilled water. Aliquots of batatasin-III stock solutions were added to petri dishes before moistening the substrates in quantities to give 0 (control), 0.1, 0.5, 1, 2 or 4 mM concentrations in the added water volume; thus the absolute quantities of batatasin-III per petri dish were higher in peat than in sand. However, solute concentrations of available batatasin-III in growth media are not known although they are more likely to be close to the added concentration in sand as opposed to peat, since sand has low absorptive capacity. Control series on filter paper were prepared as in bioassay 1. The dishes with sand and peat were allowed to equilibrate overnight (12 h) before placing the ten pre-germinated seeds. Six replicates of each batatasin-III concentration were performed for each growth substrate and root elongation was measured after 4 days.

For the bioassay using *L. sativa*, we used 1 g of meadow humus or 1 g of sand per petri dish. Growth substrates were moistened with 1.5 ml of distilled water before adding ten pre-germinated seeds. Three replicates for each batatasin-III concentration were used for each growth substrate. Root elongation was measured after three days.

Bioassay 3

In bioassay 3, we tested the interacting effect of fertilization, pH and batatasin-III concentration on mean root elongation of *Lactuca sativa*, *Phleum pratense* and *Betula pubescens*. These three species have different pH and nutrient requirements and should therefore be good indicators for the interactive effect of batatasin-III and pH or fertilization. Pre-germinated seeds were placed on petri dishes treated with concentrations of batatasin-III of 0 (control), 0.1, 0.5, 1.0 or 2.0 mM, and adjusted for pH with citric acid–sodium citrate buffer solutions at pH 4.0, 4.8 and 5.6. After adding the pH adjusted stock solutions to petri dishes, methanol was allowed to evaporate at room temperature before the bioassays were initiated. Six replicates per batatasin-III concentration and pH were performed, half of which were supplied with 0.5 ml of nutrients in form of Hoagland solution (Hoagland and Arnon 1950) modified after Asher (1977) and the remaining half supplied with 0.5 ml of distilled water to moisten the filter paper before pre-germinated seedlings were placed on it. After three days root length was measured.

Bioassay 4

In bioassay 4, we used biological material with known levels of batatasin-III (leaves and humus of *E. nigrum*) in order to study the correlation between the amount of batatasin-III measured in the biological material and the effect on mean root elongation.

Empetrum nigrum shoots and humus were sampled during July 2008 from several *E. nigrum* dominated heaths over a 10 km^2 area in Ifjordfjellet (same location as in bioassay 2). The first 2 cm of humus were collected using a metallic soil core of 1.5 cm in diameter. The pH of the sampled humus was about 4. Both shoots and humus were dried at room temperature and stored in paper bags until the experiments started.

In each petri dish, 25 mg of green dry *E. nigrum* leaves or 1 g of dried humus was placed under two Whatman no. 1 filter papers moistened with 0.5 ml (for leaves) or 1.5 ml (for humus) of distilled water. A total of 40 petri dishes with leaves and 20 petri dishes with humus were tested. Ten pre-germinated seeds of *Lactuca sativa* were placed in each petri dish. After three days root elongation was measured.

Analyses of batatasin-III from *E. nigrum* leaves and humus used in bioassay 4

Sample preparation for batatasin-III analysis of *E. nigrum* leaves and humus was performed according to the procedure described by Gallet et al. (1999) (for leaves) and Wallstedt et al. (2005) (for humus) with the following modifications. Batatasin-III was extracted from 25 mg of dried leaves or 1 g of dried humus with ethyl acetate. Extracts were evaporated to dryness with Speedvac concentrator and Rotary evaporator. Dried residue was dissolved in 1 ml of acetonitril:water = 1:1 (V:V) acidified with 0.5% of acetic acid for leaves and in 1 ml acetonitrile for humus. Leaf solutions were filtered with 0.45 µm Millipore filter.

Separation and quantification of batatasin-III was performed using gradient high performance liquid chromatography (HPLC) with DAD detection. Analyses were made with Waters Alliance chromatographic system. Detection was made using 996 Photodiode array (PDA) detector. Batatasin-III was separated on Waters x-bridge C18-5 µm

particle size column (4.6 × 150 mm) (part no. 186003116). Batatasin-III was confirmed using an internal standard, quantified using the appropriate calibration curve. Retention time was 20.8 min. Analyses were performed at 30°C. Injection volume was 20 µl with a flow rate of 1ml min^{-1}. For efficient batatasin-III determination a gradient of mobile phases was used. The mobile phase used was milliQ water (A) and acetonitrile (B), both acidified with 0.5% acetic acid. The wavelength measured was 273.2 nm. See Supplementary material Appendix 1 for chromatogram examples of the extracted batatasin-III from leaves, humus and purified batatasin-III.

Statistical analyses

All data were normally distributed and analysed using linear models in the statistical environment R. Root elongation was averaged to the mean root elongation of the 10 pre-germinated seeds per petri dish (i.e. one measure per petri dish). In bioassay 1, the response variables were either mean seedling root elongation or percentage of germinated seedlings, depending on the bioassay performed. Batatasin-III concentration was the only predictor variable. In bioassay 2, the response variable was the mean seedling root elongation and the predictor variables were growth substrate and batatasin-III concentration and their interaction. In bioassay 3, the response variable was mean seedling root elongation and the predictor variables were pH, fertilization and batatasin-III concentration as well as their interactions. In bioassay 4, the response variable was mean seedling root elongation and the predictor variable was batatasin-III concentration in leaves or humus depending on the bioassay performed. Linear models were chosen using Akaike´s information criterion (AIC) that allows for the selection of the best-fitted model for the data (Crawley 2007). For the relationship between batatasin-III content of leaves and humus and their inhibitory activity on mean root elongation, Pearson's chi squared correlation analyses were performed.

Results

Bioassay 1: Effect of batatasin-III on germination and root elongation of selected growth forms

Batatasin-III inhibited germination and root elongation of all the tested species, but the strength of this effect differed among the species (Table 1). In general, concentrations higher than 1 mM were needed to give a 50% inhibitory effect of germination (Table 1). Among the most sensitive species, we found the grass *Agrostis tenuis* and the forb *Arabidopsis thaliana* (Table 1). It is worth noting that mean tree root elongation was strongly affected by increasing batatasin-III concentration, but that seed germination of some tree species like *Pinus sylvestris* was barely affected by increasing concentrations of batatasin-III (Fig. 1). Roots of some dwarf shrubs like *Calluna vulgaris* and *Betula nana* died in concentrations above 0.5 mM (Table 1). No seeds of *Calluna vulgaris* germinated in concentrations above 1 mM, and *Vaccinium myrtillus* gemination was also highly reduced in concentrations above 1 mM (Fig. 1).

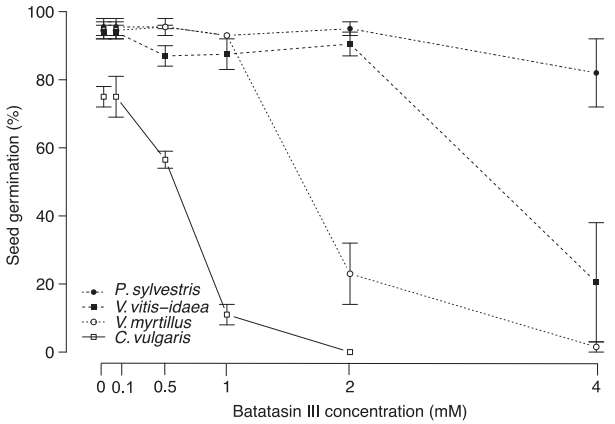

Figure 1. Effect of batatasin III on germination (%) (average ± SE) of selected species.

Bioassay 2: Effect of growth substrate on the inhibitory effect of batatasin-III

A strong interaction was found between batatasin-III, substrate type and mean root elongation on both *Lactuca sativa* and *Pinus sylvestris* (Table 2). In both cases, the inhibitory effect of batatasin-III was significantly reduced in organic soil (peat and humus) as compared to mineral soil (sand) (Fig. 2a–2b). Mean root elongation was negatively correlated with batatasin-III concentrations in sand but remained constant in peat and humus.

Bioassay 3: Effect of pH and fertilization on the inhibitory effect of batatasin-III

Mean root elongations of *Lactuca sativa*, *Phleum pratense* and *Betula pubescens* were negatively affected by batatasin-III (Table 3). However, no interactions between batatasin-III and pH or fertilization were found for any of the three species (Table 3).

Bioassay 4: Effect of *E. nigrum* leaves and humus

The measured batatasin-III concentration in leaves ranged between 0–35 mg g^{-1} of dry leaf matter (mean value 19.7 ± 10.8 (SE) mg g^{-1} of dry leaf matter). Batatasin-III concentration in humus ranged between 0–6 µg g^{-1} DW (mean value of 1 ± 1.5 (SE) µg g^{-1} DW). The values were

Table 2. Anova table from linear model from bioassay 2 showing the effect of batatasin–III concentration, growth substrate and their interaction, on the mean root elongation of *L. sativa* and *P. sylvestris*.

Species	Predictor	DF	Sum–Sq	F-value	p-value
L. sativa	Batatasin–III	1,32	71.40	34.10	<0.001
	Substrate (sand and humus)	1,32	28.80	12.54	<0.01
	Batatasin–III × Substrate	1,32	61.15	26.64	<0.001
P. sylvestris	Batatasin–III	1,146	2227.7	141.636	<0.001
	Substrate (sand, peat and control)	2,146	1040.8	520.39	<0.001
	Batatasin–III × Substrate	2,146	1070	34.014	<0.001

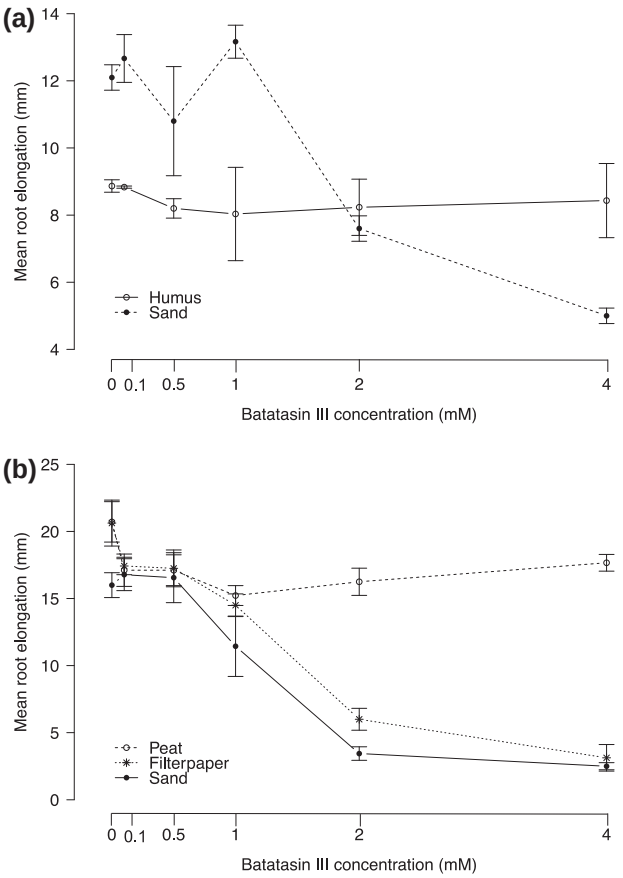

Figure 2. Effect of batatasin-III concentration (mM) in Bioassay 2 on (a) *L. sativa* mean root elongation (mm) (average± SE) in sand and humus, and on (b) *P. sylvestris* mean root elongation (mm) (average± SE) on growth substrates peat, sand and filter paper (control).

within the range of those found in other studies using the same extraction method (Nilsson et al. 1998, Wallstedt et al. 2005, Brathen et al. 2010) (see Supplementary material Appendix 1 for chromatogram examples).

The mean root elongation of *Lactuca sativa* was significantly reduced by presence of *E. nigrum* leaves in the petri dish (model estimate of –0.29 ± 0.06 (SE), $p < 0.01$, $R^2 = 0.47$) (Fig. 3). Surprisingly, the batatasin-III concentration measured in the leaves was not correlated with the mean root elongation (model estimate of –0.018 ± 0.035 (SE), $p = 0.603$, $R^2 = –0.018$) (Fig. 3).

Table 3. Anova table from linear model from bioassay 3 showing the effect of batatasin–III concentration, pH and fertilization on mean root elongation of *L. sativa*, *P. pratense* and *B. pubescens*. Only the significant interactions between the predictor variables are presented.

Species	Predictor	DF	Sum–Sq	F-value	p-value
L. sativa	Batatasin–III	1,85	5954	127.21	<0.001
	pH	1,85	109.3	2.33	ns
	Fertilization	1,85	787.4	16.82	<0.001
	pH × Fertilization	1,85	334.2	7.14	<0.001
P. pratense	Batatasin–III	1,86	8215.3	158.59	<0.001
	pH	1,86	0.6	0.01	ns
	Fertilization	1,86	193.8	3.74	<0.05
B. pubescens	Batatasin–III	1,86	1487.09	215.14	<0.001
	pH	1,86	5.83	0.84	ns
	Fertilization	1,86	12.04	1.74	ns

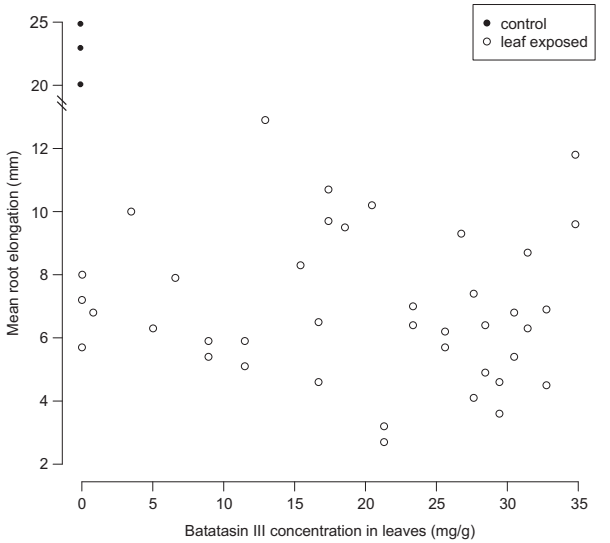

Figure 3. Effect of *E. nigrum* leaves on mean root elongation (mm) (average± SE) of *L. sativa* plotted against the batatasin-III concentration (mg g^{-1}) measured in the samples in Bioassay 4. Each dot represents one petri dish with the average mean root elongation of ten pre-germinated seeds exposed to leaves (leaf exposed) or not exposed to leaves (control).

Humus from *E. nigrum* also inhibited *L. sativa* mean root elongation, but again we found no correlation between the measured concentration of batatasin-III in humus and the variation in root elongation ($\chi^2 = 0.08$, DF = 15, $p = 0.14$). Notably, there was large discrepancy in the batatasin-III content in humus and leaves, with humus concentration being approximately 1000 × lower than in leaves.

Discussion

Our results confirm batatasin-III as a compound capable of inhibiting germination and/or mean root elongation for a range of plant species. The levels of inhibition, ranging from 0.1 to above 4.0 mM depending on the species, were mainly within the range reported by other studies (Ashina et al. 1998, Nilsson et al. 2000, Wallstedt et al. 2001b). Some woody species like *Calluna vulgaris* had a low threshold for batatasin-III. This species is known to loose dominance when competing with *E. nigrum* at northern latitudes (Tveraabak 2004). Among those more tolerant to germination inhibition by batatasin-III we find *Solidago virgaurea*, a species commonly found in *E. nigrum* heaths where it seems to germinate successfully, although few individuals survive the seedling stage (pers. obs.). Apparently humus beneath *E. nigrum* inhibits the growth of this species (Brathen et al. 2010), suggesting that other environmental factors or humus compounds reduce seedling establishment of *S. virgaurea*. Root elongation of both *Betula* species appears to be sensitive to batatasin-III. *Betula pubescens* is an important species in the Fennoscandian alpine forest ecotone. It has been suggested that allelopathy and competition

from neighbouring plants could be an important factor regulating the establishment of new individuals at the forest treeline zone (Moen et al. 2008), and that *B. pubescens* seedling growth could be inhibited by the presence of *E. nigrum* litter (Weih and Karlsson 1999). There is current concern that allelopathic substances accumulated in the soil at the tree line zone could inhibit the climate change induced upward shift in the tree line (Moen et al. 2008). Among the other studied tree species, we found *Pinus sylvestris* seeds to germinate at high levels of batatasin-III. Previous work has found a high seed germination inhibition of *E. nigrum* aqueous leaf extracts on *P. sylvestris* (Nilsson and Zackrisson 1992, Zackrisson and Nilsson 1992), however our results suggest that batatasin-III might not be the only substance responsible for this germination inhibition, but rather a mix of leaf components. Nevertheless, tree root elongation was strongly inhibited by batatasin-III, suggesting that seedlings would not survive after the germination stage in presence of batatasin-III. Hence, batatasin-III has clear allelopathic effects on germination or root elongation of plant species ranging from herbaceous to tree species dominating forest ecosystems.

We expected to find different effects of batatasin-III in the mineral vs the organic soil mainly because, through chemical extraction tests, it has been suggested that most batatasin-III remains bound to organic matter (Wallstedt et al. 2005). Our results support this prediction but go further, and prove that in organic soil batatasin-III is less available for direct uptake by the roots. Mineral substrates, on the other hand, do not retain batatasin-III, indicating that it can be easier washed away by precipitation but, at the same time, that it is more available for uptake by the roots. Consequently, the presence and depth of the organic layer are important factors when considering the effects of batatasin-III out in field conditions. What mechanisms affect the turnaround of the batatasin-III storage in soil are however still unknown.

Contrary to our expectations, pH and fertilization did not interact with the effects of batatasin-III. Generally, phenolic acids have a stronger inhibiting effect at low pH due to change in speciation, i.e. the ratio between its ionic and neutral forms (Harborne 1993). Humus under *E. nigrum* has a pH usually lower than 4.5 (Gallet et al. 1999, Brathen et al. 2010) and decreasing pH should give a stronger effect of batatasin-III (Nilsson et al. 2002, Brannas et al. 2004), mainly because of disturbance in the cell membrane integrity and fundamental processes such as ion uptake (Wallstedt et al. 2001b). However, due to batatasin-III's high acidity constant (i.e. pK_a, the acidity of common compounds and functional groups on a numerical scale), the difference in speciation of the compound between the tested pH might have been negligible and so, pH did not modify the activity of batatasin-III. Furthermore, the addition of nutrients through fertilization did not alleviate the stress caused by batatasin-III. In a restricted environment such as a petri dish, the irreversible damage to the root membrane caused by batatasin-III (Wallstedt et al. 2001b) might have been so severe that any differences in pH and fertilization would not help alleviate the phytotoxic effect.

When testing the effects of *E. nigrum* biological material, we surprisingly found no correlation between the phytotoxicity of the leaves or humus (i.e. reduction in mean root elongation) and the measured concentration of batatasin-III. Previous studies have used batatasin-III as an indicator for *E. nigrum*'s phytotoxic activity (Wallstedt et al. 1997, Nilsson et al. 1998, Nilsson and Wardle 2005), however our results suggest that the phytotoxicity of *E. nigrum* leaves and humus cannot be attributed to this substance alone. We therefore propose that there is more to the phytotoxicity of *E. nigrum* than batatasin-III. It has been described previously that batatasin-III in leaves can only explain up to 28% of the biological activity found in bioassays (Odén et al. 1992).

Batatasin-III soil values were significantly smaller than those found in leaves and were barely traceable with the HPLC. The study of allelopathic interactions in soil remains controversial due to difficulties in achieving accurate measurements of the targeted allelochemicals (Inderjit and Callaway 2003, Weidenhamer et al. 2009). We have previously observed a high chemical stability of batatasin-III (unpubl.), and so an alternative hypothesis for the low amounts of batatasin-III recovered is a chemical incorporation of batatasin-III into the humus constituents, like for example, to organic matter as shown in this study. Furthermore, soil microbial activity and temperature are known to affect the accumulation of phytochemicals in the soil (Blum 1998). The low concentrations of batatasin-III recovered from the humus might, thus, be partly explained by microbial degradation (Wallstedt et al. 2005) and/or seasonal variation in leaf allelochemical production (Nilsson et al. 1998). Most importantly, abiotic and biotic conditions determine the allelopathic potential of chemicals in the soil (Tharayil et al. 2006, Kaur et al. 2009, Inderjit et al. 2011), making it difficult to evaluate the importance of a single chemical in isolation. Nevertheless, both leaves and humus had a strong phytotoxic effect on mean root elongation even though it was not correlated to the measured content of batatasin-III.

In conclusion, batatasin-III limits germination and/or mean root elongation of a wide range of species. However, sensitivity to batatasin-III depends on the species, varying in the present study with about 40-fold, and was modified by environmental conditions such as growth substrate. The concentration of batatasin-III measured in leaves or humus below *E. nigrum* poorly predicted the growth inhibition caused by leaves and humus in bioassays. Thus, chemical analysis of batatasin-III alone is not sufficient for estimating the allelopathic effect of *E. nigrum*. Based on our findings more research is necessary for understanding and detecting the metabolites and mechanisms contributing to the allelopathic effects of *E. nigrum*.

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Supplementary material (Appendix NJB-00559 at < http://www.nordicjbotany.org/readers/appendix). Appendix 1