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Význam sekundárních metabolitů a jejich alelopatického efektu v invazní strategii *Heracleum
mantegazzianum*

Secondary metabolites, allelopathy and their significance in the invasion of *Heracleum
mantegazzianum*

Ph. D. Thesis

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Prohlášení

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Declaration

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V Praze,

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Podpis

Abstrakt

Jedna z teorií vysvětlujících invaze nepůvodních druhů rostlin předpokládá, že tyto rostliny uvolňují alelopatické látky, které dosud nebyly v invadovaných oblastech přítomné. V předkládané disertační práci jsem v sérii pokusů prováděných *in vitro* a v zahradních experimentech zkoumala alelopatii kořenových exudátů invazní rostliny *Heracleum mantegazzianum* (bolševník velkolepý) a srovnávala ji s alelopatii domácího příbuzného *H. spondylium* a dvou nepříbuzných domácích druhů. Dále jsem zkoumala vnitrodruhovou variabilitu alelopatie porovnáním 41 různých mateřských linií druhu *H. mantegazzianum*, které byly nasbírány v několika oblastech a populacích v České republice. Vedle alelopatických efektů jsem zkoumala i chemickou identitu látek za ně zodpovědných. Za účelem zjištění možné interakce alelopatických látek s půdními mikroorganismy jsem určila složení mikrobiálního společenstva pomocí mastných kyselin fosfolipidů (PLFA) a neutrálních lipidů (NLFA) na invadovaných lokalitách v České republice. Současně jsem na těchto lokalitách stanovovala abiotické půdní vlastnosti a dostupnost světla. Výsledky ukázaly, že *H. mantegazzianum* je schopné alelopaticky působit jak *in vitro*, tak v zahradních experimentech, nicméně inhibice tímto druhem způsobená je srovnatelná s efektem domácích druhů. Rozklad variability mezi oblastí, populace uvnitř oblastí a mateřské linie uvnitř populací ukázal, že největší podíl variability alelopatického efektu zkoumaného druhu se nachází na úrovni mateřských linií. Z metabolických profilů jednotlivých mateřských linií bylo možno predikovat sílu alelopatického efektu a současně z nich byly určeny a částečně identifikovány látky související s alelopatii. Složení mikrobiálních společenstev se neměnilo vlivem invaze ani dále v jejím průběhu. Nicméně, v důsledku invaze významně klesly kvalita a dostupnost světla a vzrostlo půdní pH. Ze sledovaných změn ve vlastnostech ekosystémů na invadovaných lokalitách bylo možno predikovat změny v druhové bohatosti a produktivitě domácích společenství a úspěšnosti bolševníku samotného. V souhrnu lze říct, že ačkoli jsou kořenové exudáty invazního bolševníku *H. mantegazzianum* schopné fyto toxicky inhibovat domácí kompetitory, v této vlastnosti se neliší od domácích druhů. Složení půdních mikrobiálních společenstev se také významně nezměnilo na invadovaných lokalitách. Alelopatie způsobená produkcí látek, které dosud nebyly v invadovaných oblastech přítomné, jak předpokládá teorie „novel weapons hypothesis“, pravděpodobně tedy není hlavním mechanismem invazního úspěchu bolševníku velkolepého.

Abstract

One theory concerning the invasiveness of exotic plants suggests that they exude allelopathic compounds that are novel in areas being invaded. Here, I tested for allelopathic effects of root exudates of the invasive plant *Heracleum mantegazzianum* (giant hogweed) in a series of experiments both *in vitro* and in the garden, and compared them with effects of its native congener *H. sphondylium* (common hogweed) and two less related native species. Moreover, I addressed intraspecific variability of allelopathy by comparing effects of 41 different maternal lines of *H. mantegazzianum* sampled from several areas and populations within the Czech Republic. Not only the effects, but also the identity of allelopathic compounds was investigated. In order to test also for the interaction of allelochemicals with soil microorganisms, I analysed the composition of soil microbial communities expressed as phospholipid and neutral lipid fatty acids (PLFA and NLFA, respectively) together with nutrient conditions and light availability at invaded sites in the Czech Republic. The results show that *H. mantegazzianum* is able to exert phytotoxic effects *in vitro* as well as in the garden experiment; however, its effects did not differ from the inhibition caused by the native species tested. Variation partitioning among areas, populations within areas and maternal lines within populations revealed that the highest variance in allelopathic effects lies among maternal lines. Allelopathic effects in the bioassay were predicted by metabolic profiles of different maternal lines and compounds associated with allelopathy were tentatively identified. The composition of soil microbial communities did not change either as a consequence of the invasion event or during the invasion procedure. On the other hand, the invasion event significantly reduced red/far-red light ratios and increased soil pH. The observed ecosystem changes at sites invaded by *H. mantegazzianum* predicted variation in native species richness and productivity as well as the performance of hogweed itself. Overall, although root exudates of the invader *H. mantegazzianum* are able to inhibit its native plant competitors, I found these effects to be comparable with the same effects of native species. Moreover, the composition of soil microbial communities was not substantially altered at sites invaded by the species. I therefore suggest that allelopathy through the production of unique and novel compounds, as predicted by the novel weapons hypothesis, is not very likely to be a principal driver of the invasion success of *H. mantegazzianum*.

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Introduction

Allelopathy

The term allelopathy was first used by Molisch (1937) to describe biochemically mediated interactions in plants; however, notes about this phenomenon can be found in gardening and agricultural chronicles since ancient times. In example, de Candolle (1832) suggested that the soil sickness problem in agriculture might be due to exudates of crop plants and that rotation of crops could help alleviate the problem. Since the 1960s, allelopathy was studied also in relation to ecological questions regarding patterning of vegetation and succession in old fields. One of the pioneer publications documented the inhibitory effect of volatile terpenes from aromatic shrubs in southern California on neighbouring vegetation (Muller 1966). Results drawn from this study were later deconstructed by Bartholomew (1970), who provided evidence that bare zones around aromatic shrubs were caused by increased animal trampling. Nevertheless, research in allelopathy was growing in the 1970s. Moral and Cates (1971) investigated forty plant species common in western Washington state and found most of them to contain inhibitory compounds; some of them were associated with distinct changes in ground-cover composition beneath the canopy. Elroy L. Rice described the significance of allelopathy in old-field succession (reviewed in Rice, 1979). The weedy stage that develops immediately after field abandonment lasts 3–5 years in the tall grass prairie region of Oklahoma and Kansas. Because pioneer species eliminate themselves through strong allelopathic interactions, the vegetation shifts towards the stage of annual grasses, which are tolerant to phytotoxins. Then, the annual grasses allelopathically inhibit the nitrogen fixing bacteria which postpones the ingress of species with higher nutrition demands. All this indicates that allelopathy as a phenomenon plays an important role in shaping the structure of communities and ecosystem processes. Later on, however, research into allelopathy got criticized mainly because of methodological issues (*e.g.* Harper 1977). Specifically, bioassays used to demonstrate allelopathy at the time were criticised for not representing ecological reality and not confirm to patterns of vegetation observed in the field (reviewed in Wardle et al., 1998). Consequently, allelopathy remained a domain of agricultural scientists, and ecological research into allelopathy experienced a lag-phase until the beginning of the new

millennium, when the phenomenon was brought up to explain the success of invasive plants (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004) (Fig. 1).

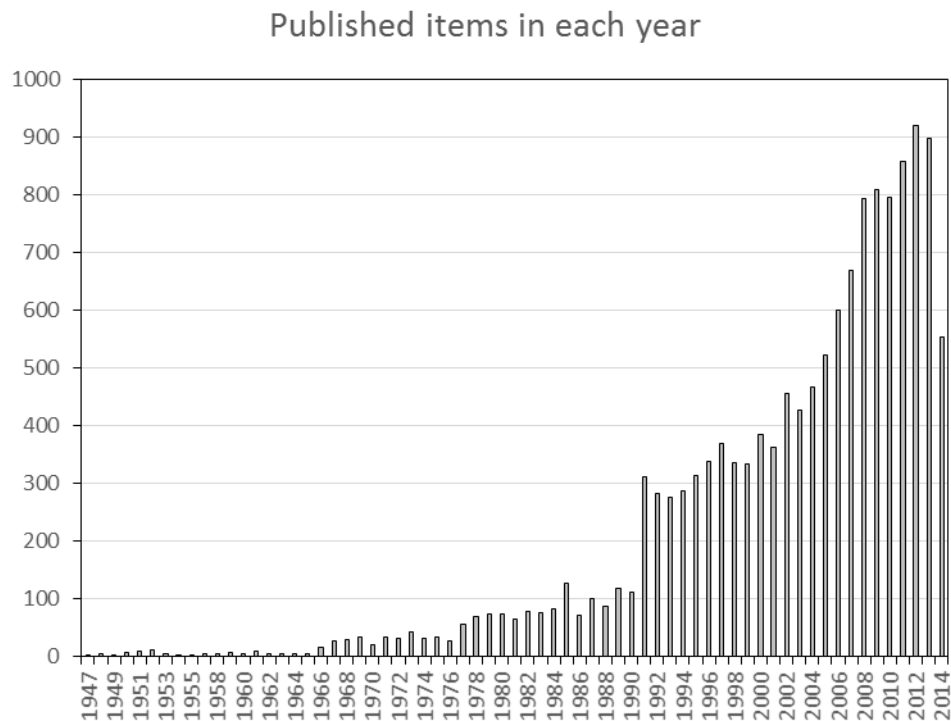


Fig. 1.1 Publications per year listed by ISI Web of Science for searches made using the string “TOPIC: (allelopat* OR phytotoxic*)”. The search was performed on 3rd October 2014.

Plant invasions

Increased global traffic causes an increased level of plant introductions (Vitousek et al. 1996). About 1% of plants introduced to new ranges become invasive (Williamson and Fitter 1996). Invasive plants often change the structure of receiving ecosystems (Levine et al. 2003), they pose a substantial threat to native species biodiversity (Vilà et al. 2011) and cause great economic losses (Pimentel et al. 2001). Explaining why the distributions of certain plant species in their new ranges differ from those in their home ranges is one of the main aims of contemporary ecology. Whether or not a plant becomes invasive depends on its traits (invasiveness) and on characteristics of the receiving ecosystem (invasibility) (Richardson and Pyšek 2006). Numerous hypotheses have been proposed to explain the success of plant invaders (Catford et al. 2009). Most of these hypotheses assume that invasive species carry certain advantages over native species that make them successful in their new ranges. The mechanisms invoked by these hypotheses include lower enemy pressure (enemy release hypothesis – Keane and Crawley 2002; Colautti et al. 2004; Joshi and Vrieling 2005),

evolutionary changes (the evolution of increased competitive ability – Blossey and Notzold 1995; Joshi and Vrieling 2005), alteration of the soil microbial community to the detriment of native species (accumulation of local pathogens hypothesis – Eppinga et al. 2006) or chemical inhibition through secondary metabolites (novel weapons hypothesis – Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004).

Novel weapons hypothesis

The novel weapons hypothesis claims that invasive species inhibit native species in new ranges through the release of secondary metabolites. The effect of invasive species is particularly strong because invasive and native species have different evolutionary histories, which makes native species, to a certain degree, innately susceptible to the effects of allelochemicals released by invaders. (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004). Biogeographical comparisons where invasive species are studied in their native and introduced ranges to compare the effects on native species from either range are therefore common (*e.g.* Prati and Bossdorf 2004; Callaway et al. 2008; Thorpe et al. 2009). In addition, the community approach, which estimates possible advantages of introduced species over native species in the introduced range (*e.g.* Del Fabbro et al. 2014), and congeneric comparison (*e.g.* Jarchow and Cook 2009 or Dostál 2011) are established ways of testing the novel weapons hypothesis.

Secondary metabolites that exert allelopathy can be released in the form of volatile compounds, root exudates, above-ground plant leachates or plant litter (Duke 2010). Released allelochemicals are indeed subject to sorption on soil particles as well as chemical and microbial decomposition (Kaur et al. 2009; Lankau 2010). Nevertheless, if sufficient concentrations are maintained in the environment, allelochemicals can inhibit other plants either directly, or the effect can be mediated by the soil biota. A growing body of evidence concerning the importance of indirect effects involving the soil microbiota has recently been accumulated (Inderjit et al. 2011; Cipollini et al. 2012; Zeng 2014). Therefore, when attempting to prove allelopathy, bioassays should be accompanied by more ecologically relevant studies done in soil and at least one of the comparative approaches discussed hereinbefore (Inderjit and Callaway 2003; Inderjit et al. 2008).

Allelopathy has been shown to drive the invasive success in several systems. For example, *Solidago canadensis*, a herbaceous perennial plant of the family *Asteraceae* native to north-eastern North America but established as an invasive plant in many other regions,

suppressed the germination of five out of seven native species in Europe (Abhilasha et al. 2008). Flowering plants of the family *Brassicaceae* native in Europe, *Alliaria petiolata* and *Brassica nigra*, have invaded forest understories in north-eastern North America at least partly due to suppression of mycorrhizal symbiosis of native plants (Callaway et al. 2008; Lankau 2008; respectively). In addition, *A. petiolata* directly inhibits the germination of native species in the introduced range, but not in its home range (Prati and Bossdorf 2004). *Fallopia bohemica*, an aggressive invader in Europe, has also been shown to be allelopathic (Murrell et al. 2011). Kim and Lee (2010) investigated nine native and nine invasive plants in East Asia and found that invasive plants exhibited higher allelopathic activity and contained higher average amounts of phenolics, which are believed to be associated with allelopathy.

Compounds identified to act as allelochemicals in invasive plants

Testing the novel weapons hypothesis entails looking for inhibitory effects of invasive plants, but studies aiming to identify chemical compounds responsible for such effects are equally important. Several studies on allelopathy in invasive plants have aimed to identify allelopathic bioactive compounds. Here, I provide a comprehensive overview of compounds and classes of compounds associated with phytotoxic or allelopathic effects in invasive plants. I searched for the string “TOPIC: (allelopat* OR phytotoxic*) AND (compounds OR identi*) AND (invasive plants)” in ISI Web of Science on 19th September 2014, which returned 151 citations. I then excluded publications not containing information on compound identity or class and publications aimed at allelopathy targeted at microorganisms. I ended up with 59 publications and extracted information on compounds identified to act as allelochemicals in 44 different invasive plants (Table 1.1).

In general, plant secondary metabolites are highly diverse, approximately 200 thousand different compounds are known. Accordingly, compounds responsible for allelopathy possess highly variable chemical features at the molecular level. Some representative structures are shown in Fig 1.2. Phenolics were considered as bioactive compounds in half of the species. Flavonoids followed, being responsible for allelopathy in quarter of the plants from the list. In about one fifth of the plants, terpenoids were identified as allelochemicals, mostly represented by sesquiterpenes, but monoterpenes were also active, and in three plants unspecified volatile organic compounds were indicated. Other classes of compounds from our list were polyacetylenes, very often substituted, alkaloids, fatty acids, substituted hydrocarbons and soluble proteins. Importantly, the named classes of compounds

are common secondary metabolites across several plant families, and they are not specifically present only in invasive plants. Macel et al. (2014), who studied metabolic profiles of six successful invaders together with their native congeners, showed that plant chemistry is highly species-specific and diverse among both exotic and native species. Nonetheless, exotic species had on average a higher total number of metabolites and more species-unique metabolites compared to their native congeners. Similarly, Cappuccino and Arnason (2006), who compared exotic plant species that are highly invasive in North America with exotics that are widespread, but non-invasive, revealed that invasive plants were more likely to have potent secondary compounds that have not been reported from plants native to North America. In conclusion, it cannot be said that a particular class of compounds makes a species more invasive. Instead, phytochemical uniqueness in terms of particular compounds or their combination seems to be an important predictor of invasive success.

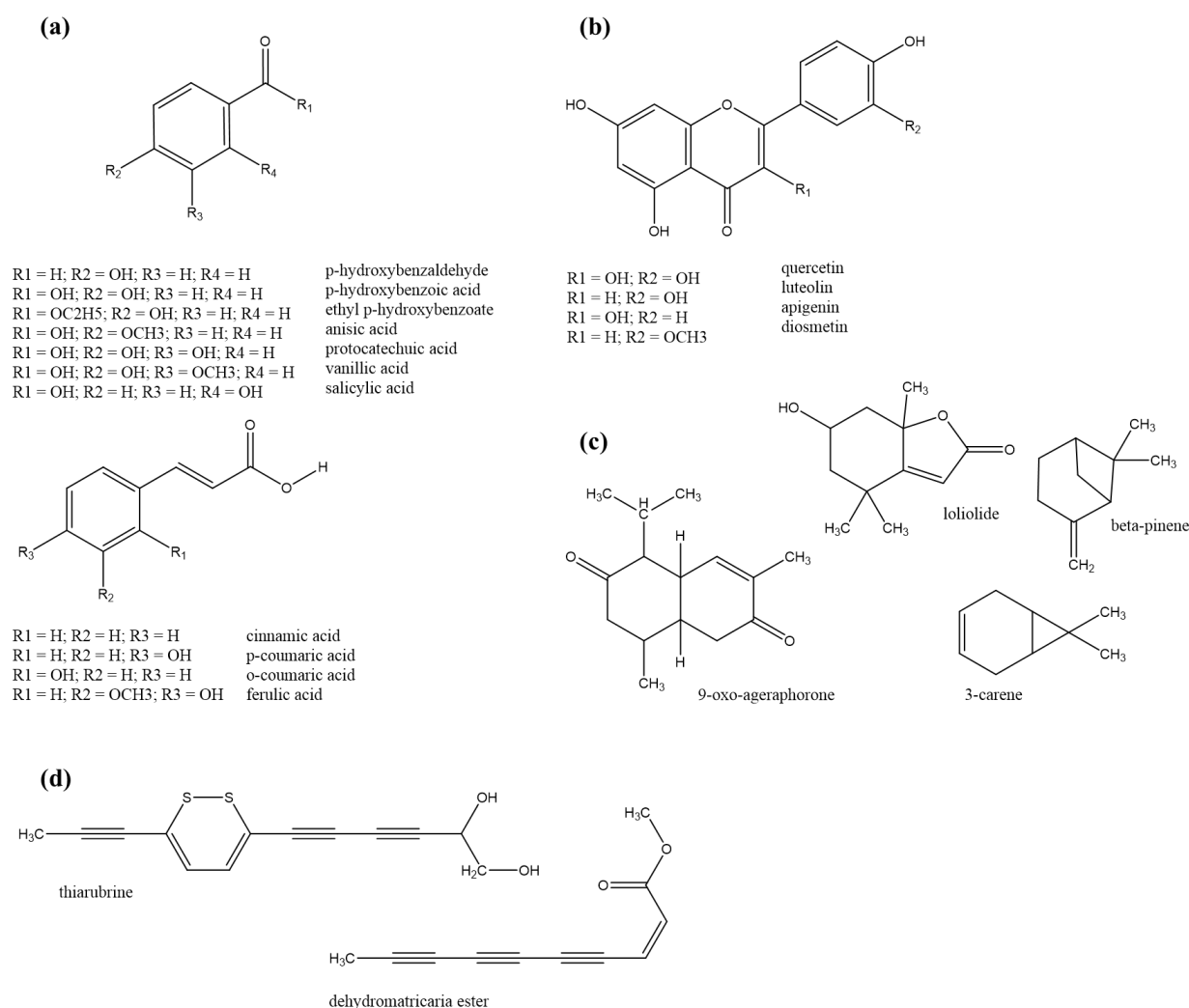


Fig. 1.2 Representative structures of most common classes of compounds which allelochemicals identified in invasive plants belong to. Phenolics (a), flavonoids (b), terpenoids (c) and polyacetylenes (d).

Table 1.1 Overview of invasive plants that have been shown to be phytotoxic or allelopathic and in which such effect was associated with particular compounds or class of compounds. I searched for the string “TOPIC: (allelopat* OR phytotoxic*) AND (compounds OR identi*) AND (invasive plants)” in ISI Web of Science on 19th September 2014, which resulted in 151 citations. I then excluded publications without information on compound identity or class and publications aimed at allelopathy targeted at microorganisms. VOCs stands for volatile organic compounds.

Nr.	Latin name	Common name	Family	Invasive range	Class of allelochemicals	Reference
1	<i>Acacia melanoxylon</i>	Blackwood	<i>Fabaceae</i>	Europe	phenolics and flavonoids	Hussain et al. (2011)
2	<i>Acroptilon repens</i>	Russian knapweed	<i>Asteraceae</i>	North America	polyacetylenes (acetylenic thiofenes)	Quintana et al. (2008)
3	<i>Ageratina adenophora</i>	Croftonweed	<i>Asteraceae</i>	Asia	phenolics	Zheng et al. (2012)
4	<i>Ageratina adenophora</i>	Croftonweed	<i>Asteraceae</i>	Asia	phenolics	Zhou et al. (2013)
5	<i>Ageratina adenophora</i>	Croftonweed	<i>Asteraceae</i>	Asia	terpenoids (mono- and sesquiterpenes)	Zhao et al. (2009)
6	<i>Ageratina adenophora</i>	Croftonweed	<i>Asteraceae</i>	Asia	phenolics and terpenoids (sesquiterpenes)	Yang et al. (2013)
7	<i>Ageratina adenophora</i>	Croftonweed	<i>Asteraceae</i>	Asia	VOCs (terpenoids, alcohols)	Zhang et al. (2012)
8	<i>Ageratum conyzoides</i>	Billy goat weed	<i>Asteraceae</i>	Asia	phenolics	Batish et al. (2009)
9	<i>Alliaria petiolata</i>	Garlic mustard	<i>Brassicaceae</i>	North America	glucosinolates	Lankau (2012)
10	<i>Alliaria petiolata</i>	Garlic mustard	<i>Brassicaceae</i>	North America	glucosinolates	Vaughn and Berhow (1999)
11	<i>Alternanthera philoxeroides</i>	Alligator weed	<i>Amaranthaceae</i>	Asia, Australia, Pacific	flavonoids and soluble proteins	Zuo et al. (2012)
12	<i>Ambrosia artemisiifolia</i>	Common ragweed	<i>Asteraceae</i>	Europe, Japan	polyacetylenes (substituted)	Shetty et al. (2007)
13	<i>Artemisia vulgaris</i>	Mugwort	<i>Asteraceae</i>	North America	terpenoids (monoterpenes)	Barney et al. (2009)
14	<i>Bonnemaisonia hamifera</i>	Bonnemaison's hook weed	<i>Bonnemaisoniaceae</i>	Atlantic	substituted hydrocarbons	Svensson et al. (2013)
15	<i>Brachiaria brizantha</i>	Bread grass	<i>Poaceae</i>	Asia	flavonoids	Kato-Noguchi et al. (2014)
16	<i>Carduus nutans</i> , <i>C. acanthoides</i>	Thistle	<i>Asteraceae</i>	America, Australia, New Zealand	polyacetylenes	Silva et al. (2014)
17	<i>Centaurea diffusa</i>	White knapweed	<i>Asteraceae</i>	North America	alkaloids	Vivanco et al. (2004); Tharayil et al. (2009)

18	<i>Centaurea maculosa</i>	Spotted knapweed	<i>Asteraceae</i>	North America	flavonoids	Weir et al. (2003); Perry et al. (2005)
19	<i>Conyza canadensis</i>	Horseweed	<i>Asteraceae</i>	Europe	polyacetylenes	Queiroz et al. (2012)
20	<i>Cuscuta hygrophilae</i>	Dodder	<i>Convolvulaceae</i>		phenolics, terpenoids and fatty acids	Khanh et al. (2008)
21	<i>Cynara cardunculus</i>	Cardoon	<i>Asteraceae</i>	America	terpenoids (sesquiterpene lactones)	Rial et al. (2014)
22	<i>Eichhornia crassipes</i>	Water hyacinth	<i>Pontederiaceae</i>	Asia	phenolics and alkaloids	Shanab et al. (2010)
23	<i>Eichhornia crassipes</i>	Water hyacinth	<i>Pontederiaceae</i>	Asia	terpenoids (sesquiterpene lactones)	Kato-Noguchi, Moriyasu, et al. (2014)
24	<i>Elodea canadensis</i> , <i>E. nuttallii</i>	Canadian waterweed, western waterweed	<i>Hydrocharitaceae</i>	Europe	phenolics	Erhard and Gross (2006)
25	<i>Fallopia japonica</i>	Japanese knotweed	<i>Polygonaceae</i>	Europe	phenolics	Dommanget et al. (2014)
26	<i>Fallopia japonica</i>	Japanese knotweed	<i>Polygonaceae</i>	Europe, North America	flavonoids and their glucosides	Fan et al. (2010)
27	<i>Fallopia sachalinensis</i>	Giant knotweed	<i>Polygonaceae</i>	Europe	phenolics	Hedenec et al. (2014)
28	<i>Helianthus tuberosus</i>	Jerusalem artichoke	<i>Asteraceae</i>	Europe	phenolics	Tesio et al. (2011)
29	<i>Heracleum sosnowskyi</i>	Sosnowsky's hogweed	<i>Apiaceae</i>	Europe	phenolics	Baležentienė (2012)
30	<i>Chrysanthemoides monilifera</i> <i>spp. rotundata</i>	Bitou bush	<i>Asteraceae</i>	Australia	terpenoids (sesquiterpenes)	Ens et al. (2009, 2010)
31	<i>Chrysanthemoides monilifera</i> <i>spp. monilifera</i>	Boneseed	<i>Asteraceae</i>	Australia	phenolics	Al Harun et al. (2014)
32	<i>Impatiens glandulifera</i>	Himalayan Balsam	<i>Balsaminaceae</i>	Europe	substituted PAHs	Ruckli et al. (2014)
33	<i>Imperata cylindrica</i>	Cogongrass	<i>Poaceae</i>	Europe, North America	phenolics and alkaloids	Hagan et al. (2013)
34	<i>Ipomoea cairica</i>	Railroad creeper	<i>Convolvulaceae</i>	Asia, North America, Europe	flavonoids	Ma et al. (2009)
35	<i>Lantana camara</i>	Big sage	<i>Verbenaceae</i>	Asia	VOCs	Zhang et al. (2009)
36	<i>Lonicera maackii</i>	Amur honeysuckle	<i>Caprifoliaceae</i>	North America	phenolics, flavonoids and their glucosides	Cipollini et al. (2008)
37	<i>Merremia umbellata</i> subsp <i>orientalis</i>	Hogvine	<i>Convolvulaceae</i>	North Asia	phenolics	Yan et al. (2010)
38	<i>Mikania micrantha</i>	Bitter vine	<i>Asteraceae</i>	Asia	terpenoids (sesquiterpenoids)	Shao et al. (2005)

39	<i>Mikania scandens</i>	Climbing hempweed	<i>Asteraceae</i>	Asia	alkaloids	Piyasena and Dharmaratne (2013)
40	<i>Oxalis pes-caprae</i>	Bermuda sorrel	<i>Oxalidaceae</i>	Europe, Australia, North America	phenolics	DellaGreca et al. (2008)
41	<i>Phragmites australis</i>	Common reed	<i>Poaceae</i>	North America	phenolics	Rudrappa et al. (2007)
42	<i>Sesbania virgata</i>	Wand riverhemp	<i>Fabaceae</i>	South America	flavonoids	Simoes et al. (2008)
43	<i>Solidago altissima</i>	Late goldenrod	<i>Asteraceae</i>	Asia, Europe	polyacetylenes	Johnson et al. (2010); Uesugi and Kessler (2013)
44	<i>Solidago canadensis</i>	Canada goldenrod	<i>Asteraceae</i>	Asia, Europe	fatty acids	Zhang et al. (2014)
45	<i>Solidago canadensis</i>	Canada goldenrod	<i>Asteraceae</i>	Asia, Europe	flavonoids	Li et al. (2011)
46	<i>Solidago canadensis</i>	Canada goldenrod	<i>Asteraceae</i>	Asia, Europe	phenolics	Yuan et al. (2013)
47	<i>Sorghum halepense</i>	Johnsongrass	<i>Poaceae</i>	Asia, America	phenolics, their glucosides and flavonoids	Liu et al. (2011)
48	<i>Spartina alterniflora</i>	Smooth cordgrass	<i>Poaceae</i>	Pacific coasts, Europe	phenolics and fatty acids	Zheng et al. (2011)
49	<i>Spartina alterniflora</i>	Smooth cordgrass	<i>Poaceae</i>	Pacific coasts, Europe	phenolics and fatty acids	Liang et al. (2012)
50	<i>Synedrella nodiflora</i>	Synedrella	<i>Asteraceae</i>	Asia	phenolics	Ghayal et al. (2008)
51	<i>Typha angustifolia</i>	Narrow-leaved cattail	<i>Typhaceae</i>	North America	phenolics	Jarchow and Cook (2009)
52	<i>Ulmus pumila</i>	Siberian elm	<i>Ulmaceae</i>	Europe, North America	phenolics	Perez-Corona et al. (2013)
53	<i>Vincetoxicum rossicum</i> , <i>V. nigrum</i>	Pale swallow-wort, black swallow-wort	<i>Apocynaceae</i>	North America	alkaloids	Gibson et al. (2011)
54	<i>Xanthium italicum</i>	Italian cocklebur	<i>Asteraceae</i>	Asia	terpenoids (sesquiterpene lactones)	Shao et al. (2012)
55	<i>Xanthium italicum</i>	Italian cocklebur	<i>Asteraceae</i>	China	VOCs	Shao et al. (2013)

Allelopathy mediated by soil biota

Apart from direct plant – plant interactions, allelopathy can be mediated by processes occurring in soil. The suppressive effect of released allelochemicals can be either further enhanced or alleviated by the soil biota. For example, bioactive (-)-catechin of the invasive shrub *Rhododendron formosanum* undergoes microbial conversion into protocatechuic acid, which exhibits higher phytotoxicity than (-)-catechin itself (Wang et al. 2013). On the other hand, Zhu et al. (2011) and Lankau (2010) suggested that the soil biota is responsible for the reduced phytotoxicity of *Ageratina adenophora* and *Alliaria petiolata*, respectively. The direction and magnitude of interference between the soil biota and allelopathic effect is likely to be context-dependent with regard to the soil community, target species or time (Bauer et al. 2012; Lorenzo et al. 2013).

Invasive plants often create positive soil feedback through interference with arbuscular mycorrhizal fungi (AMF). They can either promote AMF beneficial for their own growth, as shown for *Chromolaena odorata* and *A. adenophora* (Xiao et al. 2014), *Solidago canadensis* (Yuan et al. 2014), and *Triadica sebifera* (Paudel et al. 2014), or they may inhibit symbiotic relationships of competing native species, as shown for *A. petiolata* (Callaway et al. 2008) and *S. canadensis* (Zhang et al. 2007). In parallel, *Fallopia sachalinensis* (Hedě́nec et al. 2014) and *S. canadensis* (Zhang et al. 2009) created positive soil feedback via inhibition of soil-borne pathogens; however, *C. odorata* did so via accumulation of pathogens detrimental only to competing native species (Mangla et al. 2008). Many other invasive plants gain an advantage through unspecified alteration of soil microbial communities, as shown for *Acacia dealbata* (Lorenzo et al. 2013), *A. petiolata* (Lankau 2011; 2012), *Centaurea stoebe* (Pollock et al. 2011), *C. odorata* and *A. adenophora* (Xiao et al. 2014), and *Lespedeza cuneata* (Yannarell et al. 2011). Examples of negative soil feedback of invasive plants are much sparser. These were observed for *Sapium sebiferum*, a successful woody invader in south-eastern USA, which showed lower survival and biomass in soil collected near conspecifics than near heterospecifics (Nijjer et al. 2007). Dostál et al. (2013) found negative plant soil feedback in *H. mantegazzianum* that was temporally dependent, specifically increasing with the time the species was present at the site.

Secondary metabolites and metabolomics in allelopathy research

Being sessile organisms, plants developed secondary metabolites as a defence mechanism. Although Ernst Stahl noted already in 1888 (Stahl 1888) that these compounds function as a

defence mechanism and that their presence in plants resulted from evolutionary interactions, this was not fully accepted up until the 1960s. Instead, secondary metabolites were considered to be waste products (Wink 2003). Nowadays, the ecological role of plant secondary metabolites in defence is acknowledged, and their production is often shown to impose costs. It was hypothesized during early research into allelopathy that most phytotoxins in plants originate as metabolic by-products whose role is primarily excretory and only secondarily they are capable of inhibition of neighbouring competitors (Muller 1966; Moral and Cates 1971).

Chemical analyses of plant secondary metabolites have been performed for decades. Originally, these fell within the field of classical phytochemistry, and later on they accompanied the boom of chemical ecology that started in the 1960s (Wink 2003). Bioassay-guided fractionation, subsequent compound purification and structure elucidation represent the classical work-flow within the field. These methods allow the study of only a fraction of all metabolites, depending on the solvent and separation method used. However, plant secondary metabolites are highly diverse; approximately 200 thousand different compounds are known. Unlike sequence-based macrostructures such as DNA, RNA or proteins, small organic molecules possess highly variable chemical features at the molecular level. Analysis of the entire metabolome, the sum of an organism's synthesized metabolites, has become possible only with the development of robust analytical technologies and highly powerful data processing (Fiehn 2002).

A new era of metabolomics has started around the beginning of the new millennium. Although systematic biology benefits the most from metabolomics, untargeted metabolomics has found increasing use in ecology and environmental science. Atkinson et al. (2012), for example, used untargeted metabolomics to test the life history theory when comparing metabolic profiles of storage organs of fast- and slow-growing plants. Davey et al. (2008; 2009) described the effect of a climate latitudinal gradient on metabolic profiles of different populations of *Arabidopsis lyrata* and later tested the effect of cold on their metabolic changes directly in a manipulative experiment. Moreover, untargeted metabolomics has proven to be a good tool for identifying bioactive metabolites in the field of chemical ecology (Nylund et al. 2011; Marti et al. 2013).

Because biogeographical comparisons are established approach to researching allelopathy, and since active allelochemicals are often unknown, the field would undoubtedly benefit from employing techniques of untargeted metabolomics or metabolic profiling to compare the two sets of plants and to mine out differential compounds. Not only the

chemistry of putatively allelopathic plants, but also the change in chemistry of target species, can be studied by these techniques. The latter could be combined with other “-omic” approaches like genomics or transcriptomics to describe the mode of action of particular allelochemicals.

Aim of this thesis

In this thesis, I aimed to provide a comprehensive test of the novel weapons hypothesis in *H. mantegazzianum* invasion. Not only the allelopathic effects, but also the identity of allelopathic compounds, were investigated. Another important question in testing the novel weapons hypothesis concerns the interaction of allelochemicals with soil microorganisms. Therefore, I analysed the composition of soil microbial communities expressed as phospholipid and neutral lipid fatty acids (PLFA and NLFA, respectively) together with nutrient conditions and light availability at invaded sites in the Czech Republic. A broad range of experimental methods – *in vitro* bioassays, garden experiments, field observations - and instrumental techniques – untargeted metabolomic profiling and unknown compound characterisation, biomarker fatty acids extraction and determination - helped me to answer following research questions:

- (1) Are root exudates of *H. mantegazzianum* phytotoxic towards native plants *in vitro* as well as under ecologically more relevant conditions, such as in a garden experiment?
- (2) Does *H. mantegazzianum* have stronger phytotoxic effects than its native congener and two other, less-related native species?
- (3) How is the variation in phytotoxic effects of *H. mantegazzianum* partitioned among areas, populations within areas and maternal lines within populations?
- (4) Do the metabolic profiles of different maternal lines of *H. mantegazzianum* predict their phytotoxic effects, and what are the compounds responsible for allelopathy?
- (5) What changes occur in soil microbial communities, nutrient conditions and light availability at sites invaded by *H. mantegazzianum* for different periods of time?
- (6) Do ecosystem changes observed at sites invaded by *H. mantegazzianum* predict variation in native species richness and productivity or performance of hogweed itself?

Searching for *Heracleum mantegazzianum* allelopathy *in vitro* and in a garden experiment

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Abstract

One theory concerning the invasiveness of exotic plants suggests that they exude phytotoxic compounds that are novel in areas being invaded. For most invasive plants, however, little is known about the effects of their bioactive chemicals and how novel they are in invaded areas. From a methodological point of view, it also remains largely untested whether phytotoxicity found *in vitro* translates into allelopathic effects in more complex ecological settings. In this study, we tested for allelopathic effects of root exudates of the invasive plant *Heracleum mantegazzianum* (giant hogweed), its native congener *Heracleum sphondylium* (common hogweed) and two less related native species. We also performed chemical analyses of the invader's root exudates to identify bioactive compounds. We found that root exudates of *H. mantegazzianum* contain allelopathic compounds which are not likely to be furanocoumarins, but other as yet unidentified molecules. Allelopathy of the invader detected *in vitro* conditions and in our garden experiment did not, however, differ from the allelopathy of the native species tested. A meta-analysis of two independent garden experiments indicated significantly negative, though similar, phytotoxic effects of *H. mantegazzianum*, its native congener and *Dactylis glomerata* in the absence of activated carbon. Our study thus indicates that allelopathy by producing unique compounds, as predicted by the novel weapons hypothesis, is not a principal driver of the invasion success of *H. mantegazzianum*.

Introduction

Plant invasions are among the main concerns of contemporary ecology. Numerous hypotheses have been proposed to explain the success of plant invaders (Catford et al. 2009). Most of these hypotheses assume that invasive species carry certain advantages over native species that make them successful in new areas. The mechanisms invoked by these hypotheses include lower enemy pressure (enemy release hypothesis – Keane and Crawley 2002; Colautti et al. 2004; Joshi and Vrieling 2005), evolutionary changes (the evolution of increased competitive ability – Blossey and Notzold 1995; Joshi and Vrieling 2005), alteration of the soil microbial community to the detriment of native species (accumulation of local pathogens hypothesis – Eppinga et al. 2006) or chemical inhibition through secondary metabolites (novel weapons hypothesis). According to the last theory, allelopathy may facilitate invasions of exotic plants especially if the newcomers' bioactive compounds affect un-adapted native species (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004). Increasing attention has been paid to this theory in the last decade (reviewed in Hierro and Callaway 2003; Inderjit et al. 2008; Duke 2010). Although several studies have shown allelopathy as a possible mechanism facilitating plant invasions (Hierro and Callaway 2003; Prati and Bossdorf 2004; Cappuccino and Arnason 2006; Abhilasha et al. 2008), the extent to which novel weapons are responsible for the invasiveness of exotic species is still unknown.

The novel weapons hypothesis has usually been tested by comparing an invader's phytotoxic effects on other plants in its native range with the effects it has on other plant species in its invaded range (Callaway and Aschehoug 2000). Along with biogeographical comparisons, it is relevant to compare the composition and effects of secondary compounds of invaders with those of species in the invaded range (Cappuccino and Arnason 2006; Dostál 2011; Del Fabbro et al. 2014). This approach (community comparison *sensu* Colautti et al. 2004) is often used to estimate differences in herbivore load between invasive and native species in invaded communities. Similarly, it has been used to estimate the advantage of invaders over native relatives due to stronger allelopathic effects (discussed by Inderjit et al. 2008). Some of these studies indicate that some invaders may produce the same secondary compounds (Cappuccino and Arnason 2006) as their close relatives present in the invaded range and that these compounds have the same effects (Dostál 2011; Del Fabbro et al. 2014). This may apply to the invader *Heracleum mantegazzianum* (giant hogweed), whose congeneric species *H. sphondylium* (common hogweed) is native to Central Europe. *Heracleum mantegazzianum* is an invasive plant that is well-known for its negative impact on

the structure of native communities (Thiele and Otte 2007; Hejda et al. 2009; Dostál et al. 2013) and because it alters the properties of entire ecosystems (Thiele et al. 2010; Jandová, Klinerová, et al. 2014). It remains unclear, however, how allelopathy contributes to the invasiveness of this species (but see Junttila 1975; Junttila 1976; Myras and Junttila 1981; Wille et al. 2013). It can be argued that if an invader does not release compounds that have stronger phytotoxic effects than those released by its congeners or other less-related native species, then allelopathy through the production of unique, novel compounds, as predicted by the novel weapons hypothesis, is not very likely to be a principal driver of the invader's invasion success.

Recently, there has been a lot of debate concerning methodological issues in the field of allelopathy, and examples of incongruent results have been brought up (*e.g.* Bais et al. 2003, but Blair et al. 2006; Bertin et al. 2007, but Kaur et al. 2009). It is therefore necessary to test the repeatability of allelopathic effects by performing several independent experimental runs. Besides, many substances and plant extracts have been proved to be allelopathic only *in vitro*, and their effects under natural conditions often remain untested. Concentrations of bioactive compounds released as root exudates or aboveground biomass leachates are much lower under natural conditions than *in vitro* because they are subject to sorption on soil particles as well as chemical and microbial decomposition (Kaur et al. 2009; Lankau 2010). To obtain sufficient proof of allelopathy, *in vitro* bioassays should therefore be accompanied by more ecologically relevant approaches such as garden experiments.

Bioassays of plant exudates, leachates or extracts can provide information on the bioactivity of secondary metabolites in mixtures. Estimation of total phenolic content as a parameter of allelopathic potential can then follow (Inderjit and Nilsen 2003). Today, fractionation and chromatographic methods coupled with mass spectrometry or nuclear magnetic resonance are the methods of choice when attempting to describe the composition of particular compounds (Blair et al. 2009; Duke 2010). Separation and identification of bioactive molecules, however, remains a very difficult task. We can nevertheless distinguish among ecological treatments using techniques of metabolomics and by directly comparing fingerprint chromatograms.

In this study, we tested root exudates of the invasive plant *Heracleum mantegazzianum* and of its native congener *H. sphondylium* for allelopathic effects, both *in vitro* and in a garden experiment. In addition, we tested for allelopathy in two other, less-related native species. We also performed chemical analyses of the exudates of *H. mantegazzianum* to identify bioactive compounds. By doing so, we addressed the following

questions: (1) Do root exudates of *H. mantegazzianum* suppress the germination or root development of native plants *in vitro*? (2) Does *H. mantegazzianum* have stronger allelopathic effects than its native congener and two other less-related native species? (3) Are the allelopathic effects also detectable under ecologically more relevant conditions, such as in a garden experiment? (4) What is the chemical composition of putative allelochemicals released by *H. mantegazzianum*?

Methods

Study species

In our study, we included four species that served as a source of root exudates (*Heracleum mantegazzianum*, *H. sphondylium*, *Dactylis glomerata* and *Plantago lanceolata*), hereafter called root exudate species.

Heracleum mantegazzianum Sommier et Levier (*Apiaceae*; giant hogweed) is a monocarpic perennial with leaves up to 2.5 m long and flowering stems up to 5 m tall (Tiley et al. 1996). It is native to the Western Greater Caucasus (Russia, Georgia) and has been introduced to Europe as a garden ornamental in the 19th century (Jahodová et al. 2007). Since then it has spread to a number of European countries (Tiley et al. 1996), Canada (Page et al. 2006) and the USA (Kartesz and Meacham 1999). The species readily colonizes disturbed habitats, which offer favourable conditions for the dispersal and establishment of its seeds, but it also invades semi-natural vegetation. It is able to form extensive stands with negative effects on biodiversity (Pyšek and Pyšek 1995; Dostál et al. 2013). The chemistry of the *Apiaceae* as a whole is very complex, and *H. mantegazzianum* contains a plethora of secondary metabolites (Tiley et al. 1996). For instance, linear furanocoumarins (e.g. bergapten, psoralen, xanthotoxin), which are present in most parts of the plant, have been studied extensively as a defence mechanism against herbivory (Berenbaum 1981; Hattendorf et al. 2007) and the cause of photodermatitis in people and animals (Molho et al. 1971; Nielsen 1971; Kavli et al. 1983). Angular furanocoumarins possess remarkable fungistatic and antimicrobial properties and are responsible for below-ground protection (Fischer et al. 1978; Ivie 1978). Also present are flavonoids (Harborne 1971) and essential oils (Jain 1969).

Heracleum sphondylium L. (*Apiaceae*; common hogweed) is a mainly polycarpic perennial with stems up to 2–3 m tall. The species is widespread throughout Europe, except in the extreme north, in much of the Mediterranean region and on certain Atlantic and Mediterranean islands (Sheppard 1991). It produces flavonoids, essential oils and

furanocoumarins, which are typical of the genus *Heracleum* (Harborne 1971). There are much lower amounts of linear furanocoumarins than angular furanocoumarins (Molho et al. 1971).

Dactylis glomerata (Poaceae; cock's-foot) and *Plantago lanceolata* (Plantaginaceae; ribwort plantain) served as two less-related species releasing root exudates. These native plants have been observed to occur in communities invaded by *H. mantegazzianum* (Dostál et al. 2013).

We examined the effects of root exudates released by the invader *H. mantegazzianum*, its native congener *H. sphondylium*, and two less related native species *D. glomerata* and *P. lanceolata*. We also used the latter two species as assay species together with *Centaurea jacea*, a grassland species, and *Arabidopsis thaliana*, a widely used standard assay species.

Seeds of *C. jacea*, *D. glomerata* and *P. lanceolata* came from a commercial supplier (Planta Naturalis, Markvartice, Czech Republic), so they had no prior experience with the invader. Seeds of *A. thaliana* belonged to the Columbia-0 ecotype. Seeds and plant material of both *Heracleum* species was obtained as described below.

Root exudate collection

Root exudates of species examined for allelopathy were collected in three independent runs during the vegetative seasons of 2011 and 2012 (for the overall experimental design, see Table 1). Each time, the root exudates were obtained from an aeroponic growing system (Amazon 32, Nutriculture, Lancashire, UK) placed in a greenhouse with a 20°/10° C temperature regime at the Institute of Botany, Průhonice, Czech Republic (322 m asl; 49°99'N, 14°57'E). The collected exudates were always stored in the dark at 4°C.

In the first run carried out in May 2011, only *H. mantegazzianum* was examined. Seeds were collected in autumn 2010 from four different populations of the western part of the Czech Republic (near Lázně Kynžvart, 50°01'N, 12°38'E), mixed randomly and stored in paper bags at room temperature. Later they were cold-stratified in wet sterilized sand at 4°C in the dark for 2.5 months and then let to germinate on wet sterilized sand in the greenhouse. After two weeks of growth, the seedlings were transplanted into 50 mm mesh pots filled with 1 cm³ rockwool cubes (Grodan, Roermond, The Netherlands) and placed in a greenhouse on trays with a 0.125-strength Hoagland solution (Hoagland and Arnon 1950). After another three weeks, 32 randomly chosen seedlings were transferred to an aeroponic growing system and grown in a 0.5-strength Hoagland solution for one month. Finally, the Hoagland solution enriched with root exudates (10 L per system) was collected.

In the second run carried out in August 2011, both *H. mantegazzianum* and *H. sphondylium* were used as root exudate species. Despite the three-month-long cold stratification, the seeds of *H. sphondylium* did not start to germinate, so seedlings of both species dug out in three different populations (again from near Lázně Kynžvart) were used instead. The seedlings were thoroughly washed in water, dipped in 10% bleach for 30 min to assure sterilization and then placed in 50 mm mesh pots filled with 1 cm³ rockwool cubes. Thirty-two randomly chosen seedlings of each species were transferred to separate aeroponic systems – two per species, four in total. The plants were grown and treated as in the first run.

In the third run carried out in May 2012, both *H. mantegazzianum* and *H. sphondylium* were examined together with two native species, *D. glomerata* and *P. lanceolata*. Seeds of *H. mantegazzianum* were collected in autumn 2011 from three different populations near Lázně Kynžvart, Czech Republic, and handled as in the first run. Seeds of *H. sphondylium* were collected in autumn 2009 near Dobříš (49°47'N, 14°11'E), Czech Republic, and in autumn 2011 near Koniz bei Bern (49°55'N, 7°24'E), Switzerland, and then mixed and stored in paper bags at room temperature. Randomly selected seeds were sown into pots with a mixture of sterilized garden soil and sterilized sand in the ratio 2:1. These were left in the garden over winter to ensure cold stratification. After this four-month-long cold stratification, the pots were moved to the greenhouse, and the seeds germinated. Seeds of *D. glomerata* and *P. lanceolata* were allowed to germinate on wet sterilized sand without any additional treatment in the greenhouse. After two weeks of growth, the seedlings were washed with water, transplanted into 50 mm mesh pots filled with 1 cm³ rockwool cubes and placed in the greenhouse on trays with a 0.125-strength Hoagland solution. After another three weeks, 32 randomly chosen seedlings of each species were transferred to separate aeroponic systems – two per species, eight in total. The plants were grown and treated as described above.

In vitro bioassays

Allelopathic effects of root exudate species were examined in three independent *in vitro* bioassays on seeds of assay species *C. jacea*, *D. glomerata*, *P. lanceolata* and *A. thaliana* (for the overall experimental design, see Table 1). A 0.5-strength Hoagland solution was used as a control. Petri dishes (6 cm diameter) were fitted with sheets of qualitative filter paper (Papírna Perštejn, Czech Republic). For each assay species, ten seeds per dish were placed onto the paper, wetted with the examined or control solution and placed in a chamber with a 25°/10°C temperature and 12/12 h light and dark regime. The number of germinants was thereafter recorded twice a week; each time the respective solution was added to keep the filter paper

wet. The bioassay was terminated when no increase in the number of germinating plants was observed for at least one week. This took two weeks for *A. thaliana* and a month for the other native species. Finally, fresh root lengths of three largest seedlings per dish were determined (for *A. thaliana* under an OLYMPUS SZX12 microscope). Bioassays always started immediately after exudates were collected, and each treatment had 6 replications in the first run, and 5 replications in the second and third run.

Garden experiments

Allelopathic effects of root exudate species were assayed in two independent garden experiments using the seeds of assay species *C. jacea*, *D. glomerata* and *P. lanceolata* (for the overall experimental design, see Table 1). Both experiments were carried out in the experimental garden of the Institute of Botany, Průhonice, Czech Republic. The experiments involved treatments with root exudates, a 0.5-strength Hoagland solution being used as a control, soil microbiota treatments (added vs. sterilized) and activated carbon treatments (with vs. without). The treatments were fully crossed. First, 1-L pots were filled with a mixture of sterilized soil and sterilized sand in the ratio 2:3. The activated carbon treatment consisted of an addition of 20 mL of finely ground activated carbon powder (particle size <0.075 mm; Resorbent Ostrava, Czech Republic) per litre of the soil-sand mixture prior to filling the pots. The activated carbon was used because it is documented to have a high affinity for organic compounds, such as potentially toxic or allelopathic chemicals (Callaway and Aschehoug 2000; Inderjit and Nilsen 2003). Allelopathic effects are also documented to be modified by the presence of a living soil microbiota (*e.g.* Lankau 2010). Therefore, one month prior the start of the experiment, half of the pots were inoculated with a soil suspension (100 mL per pot). Soil from a semi-natural grassland habitat adjacent to the experimental garden in Průhonice, Czech Republic, was used to prepare the suspension (100 g soil per litre of water). Secondly, 10 seeds of the respective assay species were sown per pot. The pots were then randomly placed in beds covered with geotextile in the garden and shaded with 30% shade cloth. The plants were watered regularly with tap water, and 50 mL of root exudates or control solution per a pot were added weekly for 6 weeks in total. The number of germinants was recorded twice a week. The plants were harvested after two months. Finally, the total biomass in each pot was dried (70°C for 48 h) and weighed. There were four replicates for each treatment combination.

The first run was carried out between August 31st and October 17th 2011 and consisted of 144 pots in total (two different root exudates plus the control × three assay species × two

soil microbiota treatments × two activated carbon treatments × four replications). The second run was carried out between May 23rd and July 16th 2012 and consisted of 240 pots in total (addition of four different exudates plus control × three assay species × two soil microbiota treatments × two activated carbon treatments × four replications).

Chemical analyses of root exudates

We analysed root exudates of *H. mantegazzianum* plants used for the May 2011 and August 2011 bioassay. First, we checked for the presence of furanocoumarins, as they are typical of the *Apiaceae* family. We selected the standards bergapten and xanthotoxin because several studies have proved their presence in tissues of *H. mantegazzianum* (Berenbaum 1981; Tiley et al. 1996; Herde 2005; Hattendorf et al. 2007). Root exudates were filtered through a 390 Munktell quantitative filter paper, then extracted using SupelSelect HLB SPE 200 mg 6mL tubes (Sigma Aldrich, USA), which were selected according to the method used in Abhilasha et al. (2008), and later on tested for extraction efficiency of compounds of interest (bergapten and xanthotoxin) using a spectrophotometer (UNICAM UV/VIS UV4). The column was conditioned twice with 6 mL of methanol, equilibrated with 6 mL of water, then the sample was loaded, washed with 6 mL of water, and the absorbed substances were eluted with 6 mL of methanol. The eluent was evaporated under nitrogen flow to 2 mL, after which 1 mL was taken for further LC analyses into a new vial. The other half was evaporated to dryness before being fully redissolved in 1 mL of ethyl acetate in an ultrasonic bath for 1 hour for further GC analyses. Prior to the analyses, all samples were filtered through 0.2 µm nylon filters (Costar, USA) in a centrifuge (10 min, 5000 rpm). Initially, 500 mL of root exudates from the first collection (May 2011) were extracted; however, in order to improve analytical responses, the volume was increased to 1600 mL in the second run (August 2011). The Hoagland solution was processed in the same manner and used as a control. Commercial standards of bergapten (Aldrich) and xanthotoxin (Fluka) were directly dissolved in methanol or ethyl acetate.

One µL of the sample was injected into a GC–MS device (Varian 3400, ITS-40, Finnigan) equipped with a split/splitless injector. An HP-5 column was used for separation (30 m, 0.25 mm I.D., 0.25 µm film thickness). The temperature programme started at 60 °C and was held for 1 min in splitless mode. After 1 min, the splitter was opened, and the oven was heated to 100° C at the rate of 25° C/min, then increased to 135° C at 1° C/min and finally to 240° C at 10° C/min. The injector temperature was 240° C, and helium flowing 1 mL/min was used as the carrier gas. The solvent delay time was set to 5 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 1 scan/s under electron impact

at 70 eV, mass range 50–450 amu. The data were processed using Varian MS Work Station version 691. Peaks of bergapten and xanthotoxin were monitored using their specific even ion m/z 216.

The sample was then analysed using an Acquity UPLC system with a LCT premier XE time-of-flight mass spectrometer (Waters) with the LC Column Acquity UPLC BEH Shield RP18 (50 mm $\times$ 2.1 mm I.D., particle size 1.7 μ m, Waters) using a two-component mobile phase. The mobile phase A and B consisted of 0.1% HCOOH in water and 0.1% HCOOH in acetonitrile, respectively. The analyses were performed under a linear gradient programme (min/%B) 0/5, 1.5/5, 15/70 followed by a 1.0 min column clean-up (100% B) and a 1 min equilibration time (5% B). The total analysis time was 18 min. The column temperature was set to 40 °C, flow rate to 0.4 mL/min, and the injection volume was 5 μ L. The mass spectrometer operated in the “W” mode with capillary voltage set at +2800 or -2500 V, cone voltage +40 or -40 V, desolvation gas temperature, 350 °C; ion source block temperature, 120 °C; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h; ion guide 1 and 2 RFs, 200 and 400 V, respectively; hexapole RF, 150 V. The signal was acquired with a scan time of 0.1 s; interscan delay was 0.01 s (0.1 s for lock spray and voltage switch). Mass accuracy was kept below 5 ppm using lock spray technology with leucine enkephalin as the reference compound (2 ng/ μ L, 5 μ L/min). The UV/visible detection was carried out from 194 to 700 nm. The data were processed using MassLynx V4.1 software (Waters).

To evaluate the data, we monitored the peaks of bergapten and xanthotoxin using their specific even ion m/z 216 in the GC-MS chromatograms. We also searched for psoralen, bergapten, imperatorin, petroselinic acid, their glucosyl- and also glucosyloxy-derivatives in both ionization modes using the masses calculated by MassLynx software based on respective molecular formulae in the UPLC-TOF-MS chromatograms. In addition, we compared UPLC-TOF-MS chromatograms of root exudates and the Hoagland solution that served as the control in both ionization modes and searched for compounds present only in the exudate samples. The molecular formulae of these compounds within the error range were calculated by MassLynx software based on accurate mass and isotopic pattern recognition. Each suggested molecular formula was matched with putative structures using the Reaxys (Reaxys 2014) and KNapSAcK (KNapSAcK 2014) databases.

Statistical analyses

First, the analyses were performed separately for each run. *In vitro* bioassay responses of *C. jacea*, *D. glomerata*, *P. lanceolata* were analysed together, and that of *A. thaliana* was

analysed separately. In the analysis of the number of germinated seeds, binomial models were fitted and subsequently substituted by quasibinomial models to avoid problems with overdispersion (Crawley 2007). Components of the model were exudates in the case of *A. thaliana* and exudates, assay species and their interaction in the case of the other native assay species. When necessary, significant effects were further examined by treatment contrasts. Root length (entered as a mean of the three largest seedlings per Petri dish) was analysed by a one-way ANOVA in the case of *A. thaliana*. In the case of the other native assay species, it was analysed by a two-way ANOVA with the model components exudates, assay species and their interaction. Post-hoc comparisons (Tukey contrasts) were applied to examine significant effects. In addition, the responses of *C. jacea*, *D. glomerata* and *P. lanceolata* were analysed together with the only component of the model being exudates to evaluate the general impact of root exudates.

Germination in the garden experiment was analysed using quasibinomial models, biomass using linear models. Initially, a full model including all components (exudates, assay species, soil microbiota, activated carbon) and their interactions was constructed. Non-significant components were then removed to obtain the minimum adequate model. When necessary, significant effects were further examined by treatment contrasts.

Secondly, we carried out a within-study meta-analysis to obtain a summary effect of root exudate species across different experimental runs. We expressed effect size as the standardized mean difference (Hedge's g):

$$g = \left(1 - \frac{3}{4df - 1}\right) \left(\frac{\bar{X}_{\text{exudates}} - \bar{X}_{\text{control}}}{S_{\text{within}}}\right)$$

where df is the degrees of freedom used to estimate S_{within} (*i.e.*, the within-groups standard deviation, pooled across groups), which for two independent groups is n_1+n_2-2 (Borenstein et al. 2009). $\bar{X}_{\text{exudates}}$ and $\bar{X}_{\text{control}}$ are means of response variables of plants in Petri dishes or garden pots with added exudates and Hoagland solution, respectively. For *in vitro* bioassays, the response variable was a product of the number of germinated seeds and of the mean of root lengths of three largest seedlings per a Petri dish. A summary effect was calculated separately for each root exudate species (*e.g.*, a summary effect for *H. mantegazzianum* included runs performed in May 2011, August 2011 and May 2012), and was obtained separately for the assay species *A. thaliana* and for the other native assay species. For garden experiments, harvested biomass was used as the response variable. Effect sizes were

calculated separately for pots with and without addition of activated carbon, and for pots with sterile and non-sterile soil.

Prior to the analyses, all dependent variables were inspected and, if necessary, data were log- or square-root-transformed to meet the assumptions of homoscedasticity and normality of residuals. We used R 2.14.0 (R Development Core Team 2011) to carry out the ANOVA, linear models, generalized linear models and within-study meta-analysis (*metafor* package; Viechtbauer 2010).

Results

In vitro bioassays

We found a strong allelopathic effect of *H. mantegazzianum* root exudates in the May 2011 *in vitro* bioassay (Table 2). The effect of *H. mantegazzianum* exudates on the germination of *C. jacea*, *D. glomerata* and *P. lanceolata* was strongly negative ($F = 16.03$; $P < 0.001$) and species-specific ($F = 8.25$; $P < 0.01$; Table 2). The effect of the invader's exudates on the root length of *C. jacea*, *D. glomerata* and *P. lanceolata* was also significantly negative ($F = 211.977$; $P < 0.001$), however, this effect was not species-specific ($F = 2.345$; NS; Table 2). Only two out of sixty seeds of *A. thaliana* germinated upon the addition of *H. mantegazzianum* root exudates ($F = 143.73$; $P < 0.001$), and no roots developed at all (Fig. 2).

During the second run, in the August 2011 *in vitro* bioassay, we observed no effect of *H. mantegazzianum* (Table 2, Fig. 1 and 2). On the other hand, *H. sphondylium* exudates significantly stimulated the root length of *A. thaliana* ($F = 6.543$; $P < 0.05$). In this bioassay, the responses of native plants were not species-specific (Table 2).

During the third run, in the May 2012 *in vitro* bioassay, none of the root exudates affected the number of germinants or the root length of *A. thaliana* (Table 2; Fig. 2). The number of germinants and the root length of native species was also unaffected by the root exudates of *H. mantegazzianum* (Fig. 1). The number of germinants of native species was, however, stimulated by *H. sphondylium*, *D. glomerata* and *P. lanceolata* ($F = 6.01$; $P < 0.001$; Fig. 1). All these effects were species-specific (Table 2).

The summary effect sizes did not indicate significant allelopathic effects of any exudate species included in the experiment. This result was found regardless whether *A. thaliana* or all three native species were considered as assay species (Fig. 3).

Garden experiments

The number of germinated seeds and the biomass of assay species were not affected by root exudate species in the garden experiment carried out in 2011 (Table 3). Only *Dactylis glomerata* biomass was significantly suppressed by the root exudates of both the invasive *H. mantegazzianum* and the native *H. sphondylium* in sterile soil ($F = 2.95$; $P = 0.023$); however, this effect diminished in non-sterile soil (Fig. 4a). Otherwise, we observed only differences in growth among our assay species, and *P. lanceolata* biomass was stimulated by the addition of activated carbon regardless of the root exudate treatment.

In the garden experiment of 2012, the number of germinated seeds was not affected by any of the root exudate species (Table 3). The addition of activated carbon, however, suppressed the germination of native species regardless of the root exudate treatment ($F = 4.43$; $P = 0.036$). Neither the exudates of invasive *H. mantegazzianum* nor those of native *H. sphondylium* had any effect on the biomass of the assay species. Exudates of *D. glomerata* nevertheless did suppress the biomass of *C. jacea* in soil without activated carbon ($F = 2.70$; $P = 0.004$); however, the addition of activated carbon inverted the effect (Fig. 4b). Moreover, *P. lanceolata* exudates significantly negatively affected the biomass of the species itself ($F = 5.72$; $P < 0.001$). Furthermore, activated carbon addition stimulated the biomass of *D. glomerata* and *P. lanceolata* regardless of the exudate treatment ($F = 11.71$; $P < 0.001$).

Overall, there was a significantly negative effect of root exudates of *H. mantegazzianum* and of two out of three native species in the absence of activated carbon. An additional test, however, did not prove the invader to have stronger phytotoxic effects than the natives (z-value = -0.046; $P = 0.963$). After addition of activated carbon, the negative effects of all exudate species disappeared (Fig. 5a). Furthermore, the summary effect sizes did not indicate significant allelopathic effects of any of the exudate species whether or not the assay species were grown in non-sterile soil (Fig. 5b).

Chemical analyses of root exudates

The peaks of bergapten and xanthotoxin that were monitored using their specific even ion m/z 216 in the GC-MS chromatograms were not detected in the root exudates, although the detection limit of the whole method based on analysis of standards was about 20 ng/L. Accordingly, the monoisotopic weights of psoralen, bergapten, imperatorin, petroselinic acid, their glucosyl- and also glucosyloxy-derivatives in both ionization modes in the UPLC-TOF-MS chromatograms were not present either. The compounds that were present only in the exudate samples from the collection used for the May 2011 bioassay and not in the control

(Fig. 6) are listed in Table 4. The root exudates from the collection used for the August 2011 bioassay showed very little difference from the control, and we failed to detect any ions in the exudates which would not be present in the control. The UPLC-TOF-MS peak annotation process that included molecular adduct identification, molecular formula calculation and database matching has not allowed a full identification of compounds of interest, however, the molecular formulae within the error range were calculated based on accurate mass and isotopic pattern recognition in both ionization modes (calculations based only on one ionization mode are marked; Table 4). The database search has not revealed any compound previously reported from any *Heracleum* species. Nevertheless, compounds C17H26O4 and C27H36O5 were reported in species from the *Apiaceae* family as derivatives of sesquiterpenes daucanes. Compound C17H26O4 was reported either as siol acetate in *Sium latifolium* (Casinovi et al. 1983) and *S. latijugum* (Pandita et al. 1984) or as 11-(acetyl)torilolone in *Daucus carota* (Yi et al. 2009), whereas compound C27H36O5 may represent a trisubstituted daucane with angeloyloxy and benzoyloxy moiety that has been reported from *Ferula communis* (Miski and Mabry 1985). More research is needed to assign bioactivity to these compounds and to clarify their structure.

Discussion

In this study, we have demonstrated that root exudates of *H. mantegazzianum* are able to exert phytotoxic effects. These effects were detected not only in *in vitro* but also under garden conditions as confirmed by a meta-analytical approach. However, phytotoxicity was not consistently found across all experimental runs, and this large variation resulted in a non-significant summary effect of *in vitro* experiments. In some cases of garden experiment, the effects manifested in the absence of living soil microbiota only. Therefore, it remains unclear to what extent are competitive interactions of *H. mantegazzianum* with native species determined by allelopathy.

The identity of the compounds involved in allelopathy of the invader remains unknown, but we can rule out the furanocoumarins. Furanocoumarins had previously been indicated as possible agents responsible for allelopathy (Baskin et al. 1967; Macías et al. 1993; García et al. 2002). Junttila (1976), who studied inhibitory effects of a seed extract of *H. laciniatum*, reported three bioactive fractions including furanocoumarins to suppress germination of assay species although other unknown compounds were also involved. In the highly phytotoxic exudates used for the May 2011 bioassay, we found compounds that were

not previously reported from a *Heracleum* species; some of them, however, possibly represent sesquiterpene derivatives related to daucanes, which have been reported from different species of the *Apiaceae* family (Casinovi et al. 1983; Pandita et al. 1984; Miski and Mabry 1985; Yi et al. 2009). As we do not know the identity of the phytotoxic compounds released by the invader, we cannot say whether they are novel and unique in invaded communities, as the novel weapons hypothesis predicts (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004). A further investigation is thus needed to prove the phytotoxicity of the candidate compounds and reveal their chemical identity.

Regardless whether bioactive compounds of the invader differ from allelopathic compounds of native species, they all had a significantly negative effect on the performance of assay species used in the garden experiment. Importantly, the allelopathy of the invader did not differ from the allelopathy of its native congener or from that of less-related native species. Although Cappuccino and Arnason (2006) found that some invasive plants are phytochemically unique in their new habitats based on a survey of the literature, Lind and Parker (2010) found no difference in generalist insect herbivore preference between 19 invasive and 21 co-occurring native plant species. However, there is no such study of a similar scale in the field of plant-plant interactions. Allelopathy is mostly studied in invasive plants, so we lack information about allelopathy in native species in their native ranges. Our results nevertheless corroborate those of Del Fabbro et al. (2014) who investigated the effect of three invasive species on germination of native species in a field study and found that invasive species do not suppress seed germination of native species more than species from native plant communities. Overall, we assume that mechanisms other than allelopathy through the production of unique compounds, as predicted by the novel weapons hypothesis (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004), are more likely to explain the invasion success of *H. mantegazzianum*.

The rapid spread (Pergl et al. 2011) and strong impact of *H. mantegazzianum* on invaded communities (Jandová, Klinerová, et al. 2014) can stem not only from different properties of the invader, but also from attributes of invaded areas. The species has an enormous reproductive capacity. It can produce 20000 seeds per individual on average (Perglová et al. 2006). Seeds then play an important role in long-distance dispersal, being an important component of population dynamics of invasive species (Pergl et al. 2011). Moreover, Jandová et al. (2014) showed that *H. mantegazzianum* considerably alters the amount of light available to native species by reducing photosynthetically active radiation and red/far-red ratio in stands it invades. The decreased light levels correlated with observed

decreases in native species richness. Moreover, Müllerová et al. (2005) identified the lack of appropriate landscape management associated with disturbances and favourable climatic conditions as the probable reasons for the rapid spread of the species. All these factors, together with allelopathy found in this study, can contribute to the vast invasiveness of *H. mantegazzianum*.

Several experimental caveats should be borne in mind when drawing conclusions based on results of our study. Firstly, we only used the polar fraction of exudates. Since the solvent present in the root environment is the soil solution that is very close to water and also of high polarity, we decided to study exclusively the polar fraction of root exudates. It may be true that if we had included the non-polar fraction by employing some organic solvent, we would have found out more metabolites; however, these would not necessarily be ecologically relevant for our study. Secondly, we bioassayed the root exudates at only one concentration (that resulting from the collection of material from our growing systems). We therefore do not know whether and how different concentrations would affect our findings. However, our objective was not to estimate the amount of root exudates produced or to estimate the proportion of bioactive compounds present. Finally, our results could have been influenced by using a soil microbiota from a single source in the garden experiment. Nonetheless, it is unlikely that using a soil microbiota from multiple sources would significantly affect our conclusions. Dostál et al. (2013) searched for allelopathic effects of hogweed seedlings cultivated in soil from 20 different sites, including both invaded and uninvaded ones. They failed to prove phytotoxicity of hogweed plants, irrespective of the soil origin.

On the other hand, our study provides a comprehensive and repeated test of the novel weapons hypothesis on the example of one of the worst invasive species in Europe. We performed a series of experiments both *in vitro* and in the garden. In our garden experiments, we considered several factors which had previously been found to modify phytotoxic effects, such as activated carbon and a living soil microbiota. Previous studies have mostly considered allelopathy merely as a species trait, and when intraspecific differences were tested, they focused only on biogeographical comparisons (but see Lankau et al., 2009). Our results indicate that the allelopathic effect of *H. mantegazzianum* exudates in the species' introduced range is not a constant trait, but a highly variable one. This finding has prompted a more detailed evaluation of different populations and genotypes, which has revealed variation among genotypes (Jandová et al., preliminary results). Future research is needed to shed light on variation in allelopathy of invasive species in their introduced ranges.

To conclude, though root exudates of the invader *H. mantegazzianum* are able to inhibit the performance of native plant competitors, we found these effects to be comparable with the same effects of native species. We therefore suggest that allelopathy through the production of unique and novel compounds, as predicted by the novel weapons hypothesis, is not very likely to be a principal driver of the invasion success of giant hogweed.

Table 2.1 Overall experimental design of the study. Root exudate species are *H. mantegazzianum* (HM), *H. sphondylium* (HS), *D. glomerata* (DG) and *P. lanceolata* (PL); Hoagland's solution was always used as a control (C). Assay species are *A. thaliana* (AT)¹⁾, *C. jacea* (CJ), *D. glomerata* (DG) and *P. lanceolata* (PL).

	May2011	Aug2011	May2012
Root exudate species	HM + C	HM, HS + C	HM, HS, DG, PL + C
<i>In vitro</i> assay species	AT ¹⁾ , CJ, DG, PL	AT ¹⁾ , CJ, DG, PL	AT ¹⁾ , CJ, DG, PL
Garden assay species	-	CJ, DG, PL	CJ, DG, PL
Cultivation of root exudate species started from	seeds	seedlings	seeds
<i>In vitro</i> bioassay	+	+	+
Garden experiment	-	+	+
Chemical analyses	+	+	-

¹⁾ The assay species *A. thaliana* was used only in *in vitro* bioassays

Table 2.2 Effects of root exudates, assay species and their interaction on the response parameters of native assay species (*C. jacea*, *D. glomerata* and *P. lanceolata*) and effects of root exudates on the response parameters of *A. thaliana* throughout the three independent *in vitro* bioassays (May 2011, August 2011, May 2012). The number of germinated seeds was analysed using Analysis of Deviance, root length using Analysis of Variance. *F*-values and levels of significance (NS: nonsignificant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$) are given.

	May 2011		August 2011		May 2012	
	Germinated seeds	Root length	Germinated seeds	Root length	Germinated seeds	Root length
Native assay species						
Exudates	16.03***	211.977***	0.99 ^{NS}	2.253 ^{NS}	6.01***	7.648***
Assay species	15.02***	0.690 ^{NS}	30.41***	13.179***	74.82***	19.439***
Exudates:assay species	8.25**	2.345 ^{NS}	0.80 ^{NS}	0.687 ^{NS}	2.65*	3.412**
Assay species <i>A. thaliana</i>						
Exudates	143.73***	- ¹⁾	3.25 ^{NS}	6.543*	2.26 ^{NS}	2.858 ^{NS}

¹⁾ No roots developed at all.

Table 2.3 Overview of minimum adequate models showing significant effects explaining the number of germinated seeds and biomass of assay species in garden experiments of 2011 and 2012. The effects were selected out of the components exudates, assay species, soil microbiota, activated carbon and their interactions. The number of germinated seeds was analysed using Analysis of Deviance, root length using Analysis of Variance

	<i>F</i> -value	<i>P</i> -value	R ²
Garden experiment 2011			
<i>Number of germinated seeds</i>			
Assay species	54.55	<0.001	0.4050
<i>Biomass</i>			
Exudates	1.42	0.25	0.0058
Assay species	166.59	<0.001	0.6788
Soil microbiota	1.40	0.24	0.0029
Activated carbon	7.07	0.009	0.0144
Exudates:assay species:soil microbiota	2.95	0.023	0.0241
Garden experiment 2012			
<i>Number of germinated seeds</i>			
Assay species	110.97	<0.001	0.4579
Activated carbon	4.43	0.036	0.0091
<i>Biomass</i>			
Exudates	3.62	0.007	0.0238
Assay species	145.24	<0.001	0.4782
Activated carbon	11.71	<0.001	0.0193
Exudates:assay species	5.72	<0.001	0.0754
Exudates:assay species:activated carbon	2.70	0.004	0.0388

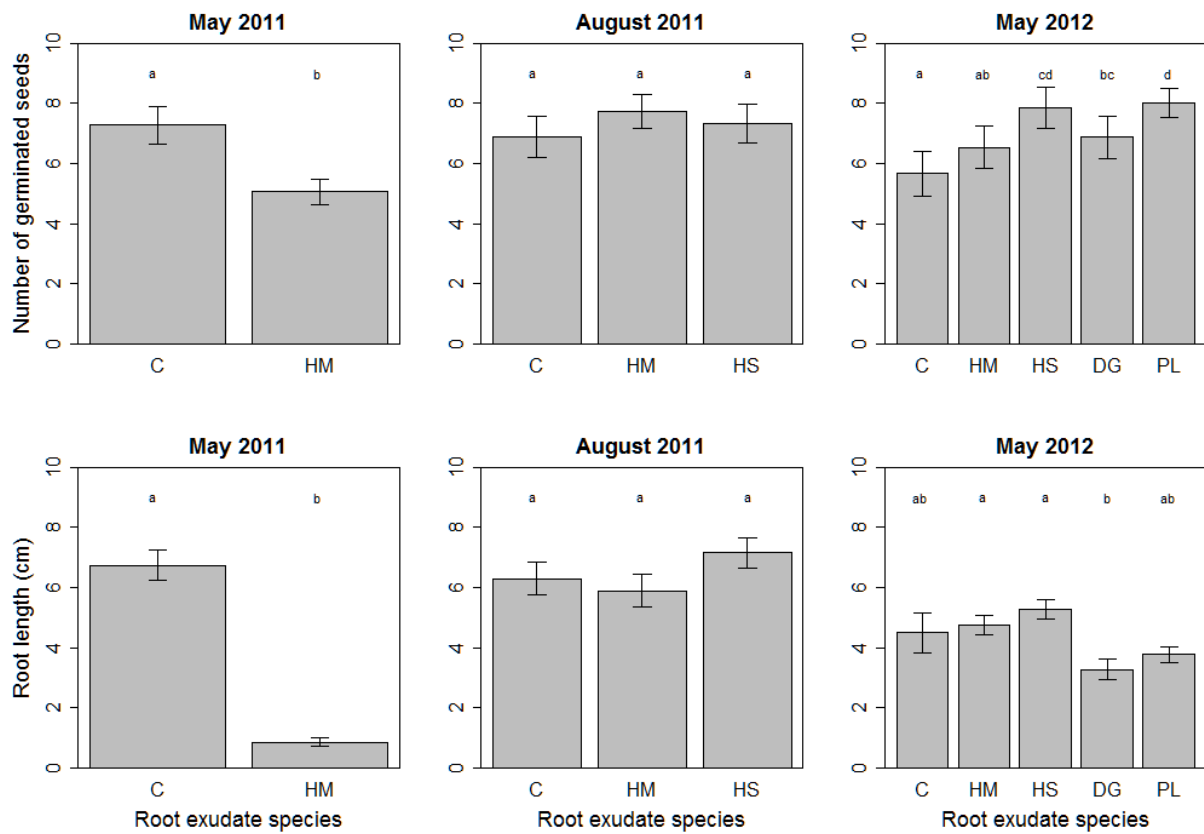


Fig. 2.1 Native assay species (*C. jacea*, *D. glomerata* and *P. lanceolata*) responses to root exudates of invasive (HM) and native (HS, DG, PL) plant species in three independent *in vitro* bioassays (May 2011, August 2011, May 2012). Means $\pm$ SE of the number of germinated seeds and of root length are displayed. Columns marked by the same letter are not significantly different ($P < 0.05$). C stands for control (Hoagland solution), HM for *H. mantegazzianum*, HS for *H. sphondylium*, DG for *D. glomerata* and PL for *P. lanceolata* (root exudate species)

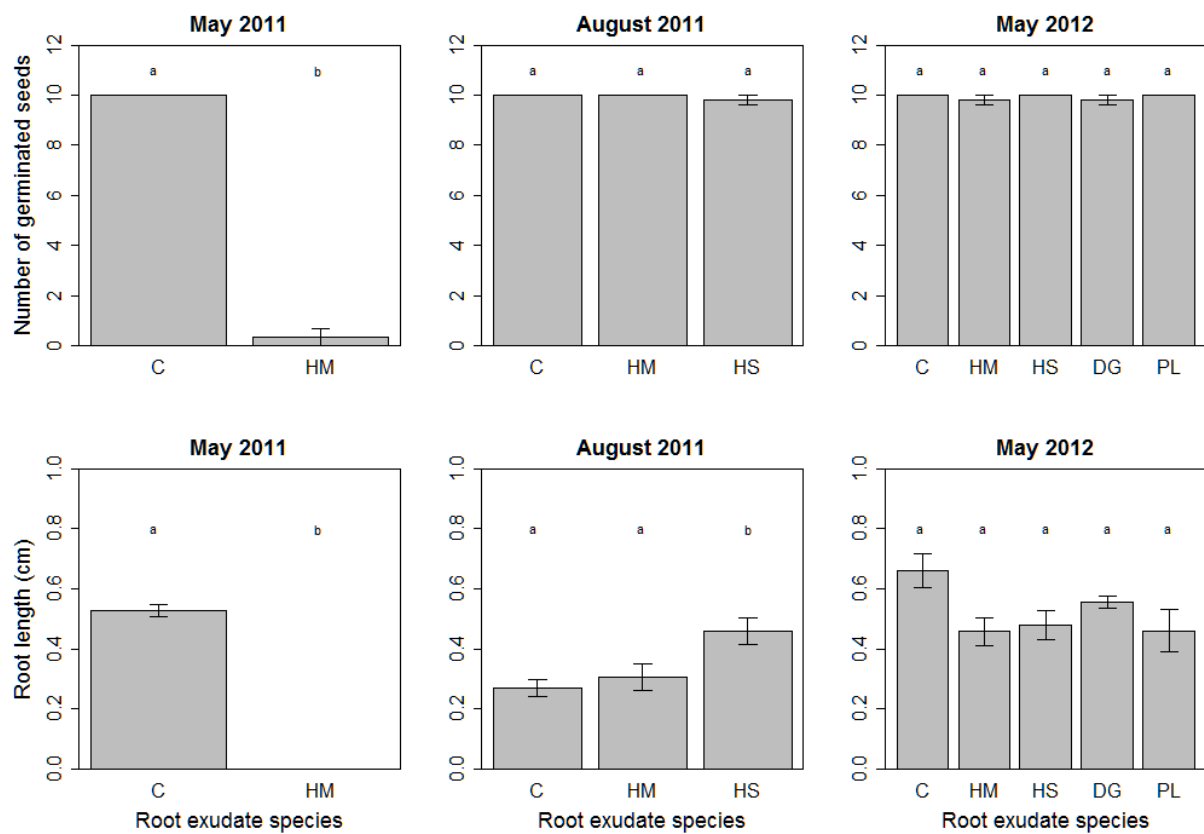


Fig. 2.2 Responses of *A. thaliana* to root exudates of invasive (HM) and native (HS, DG, PL) plant species in the three independent *in vitro* bioassays (May 2011, August 2011, May 2012). Means $\pm$ SE of the number of germinated seeds and of root length are displayed. Columns marked by the same letter are not significantly different ($P < 0.05$). C stands for control (Hoagland solution), HM for *H. mantegazzianum*, HS for *H. sphondylium*, DG for *D. glomerata* and PL for *P. lanceolata* (root exudate species)

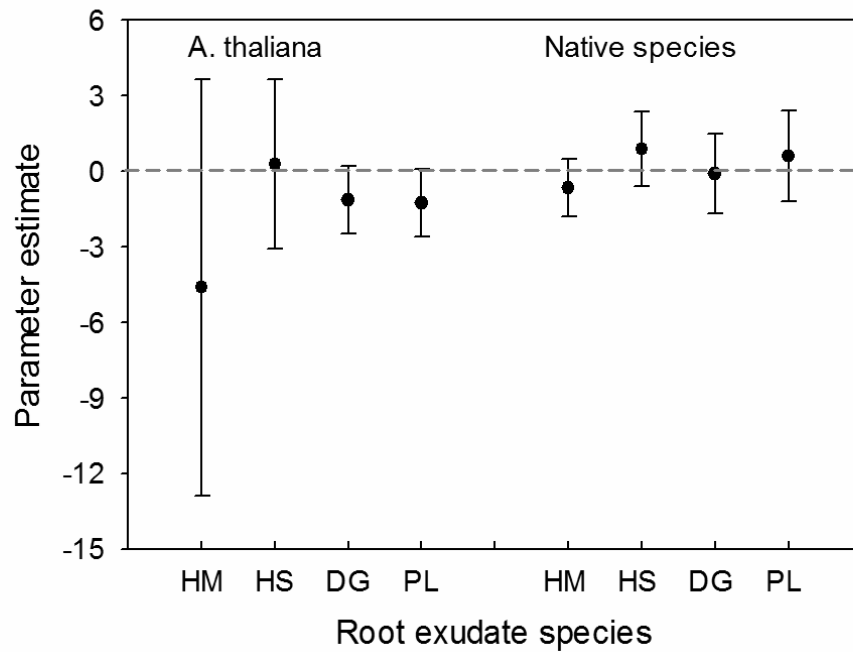


Fig. 2.3 Means and 95% confidence intervals of parameter estimates (standardized mean difference) of *in vitro* bioassays. Negative values indicate lower values of the response variables (a product of the number of germinated seeds and mean root lengths of three largest seedlings per Petri dish) when treated with respective root exudates (HM, HS, DG, PL) than when treated with Hoagland's solution used as a control. Effect sizes were calculated separately for *A. thaliana* and for the native species. None of the mean effect sizes significantly ($P < 0.05$) differed from zero (indicated by the dotted line). HM stands for *H. mantegazzianum*, HS for *H. sphondylium*, DG for *D. glomerata* and PL for *P. lanceolata* (root exudate species).

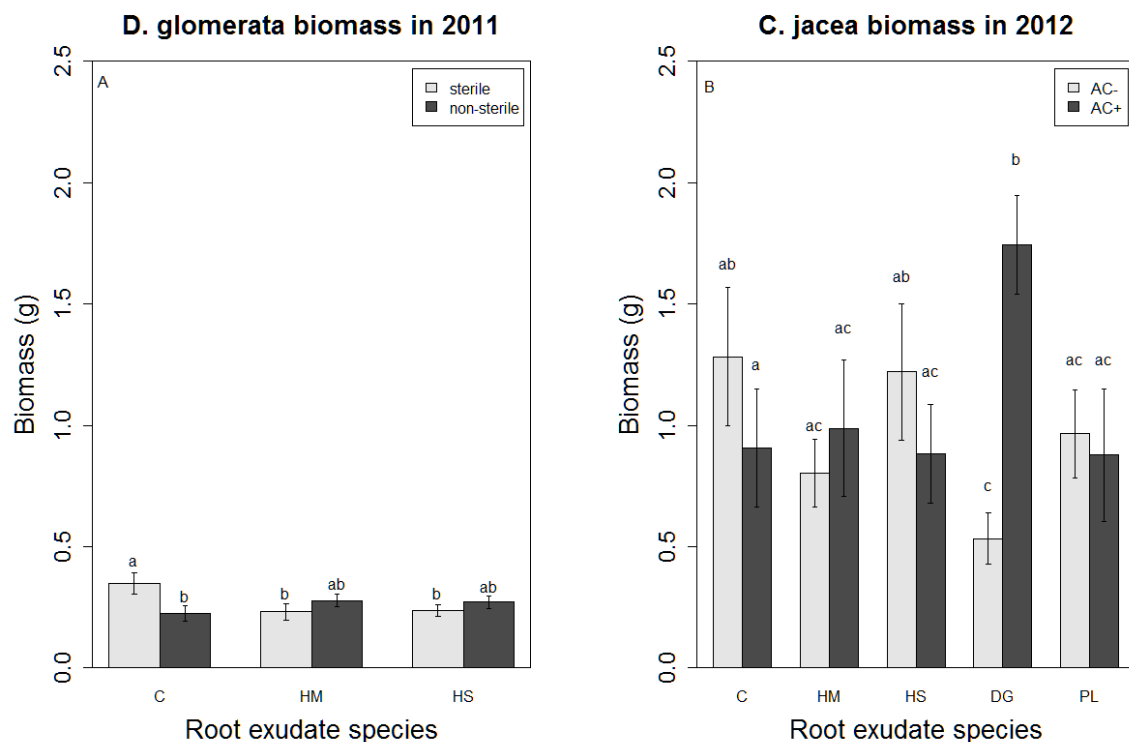
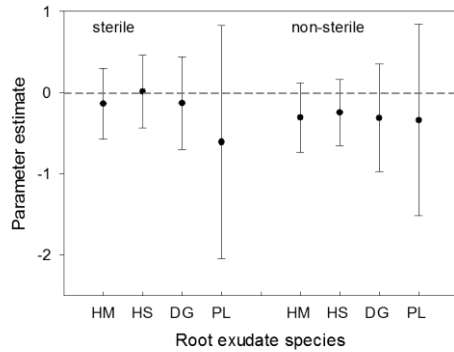


Fig. 2.4 Effects of root exudates, the soil microbiota and activated carbon on biomass of assay species in garden experiments. Means $\pm$ SE are displayed. C stands for control (Hoagland solution), HM for *H. mantegazzianum*, HS for *H. sphondylium*, DG for *D. glomerata* and PL for *P. lanceolata* (root exudate species). A) *D. glomerata* biomass was inhibited by root exudates of *Heracleum* species in 2011 in sterile soil but not in non-sterile soil. B) *C. jacea* biomass was inhibited by root exudates of *D. glomerata* in 2012, and activated carbon obviated the effect.

A



B

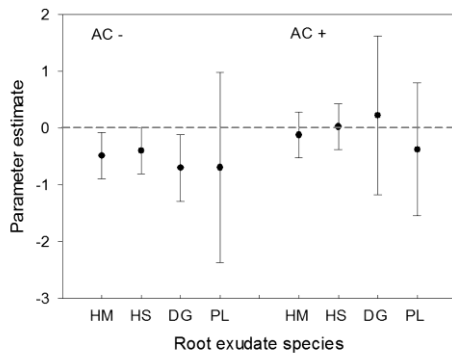


Fig. 2.5 Means and 95% confidence intervals of parameter estimates (standardized mean difference) of garden experiments. Negative values indicate lower values of the response variables (biomass of assay species) when treated with respective root exudates (HM, HS, DG, PL) than when treated with Hoagland solution used as a control. Effect sizes were calculated separately for pots with and without addition of activated carbon (A) and for pots with sterile and non-sterile soil (B). Effect sizes significantly ($P < 0.05$) differed from zero (indicated by the dotted line) for all root exudate species but *Plantago lanceolata* in the absence of activated carbon (in A). HM stands for *H. mantegazzianum*, HS for *H. sphondylium*, DG for *D. glomerata* and PL for *P. lanceolata* (root exudate species).

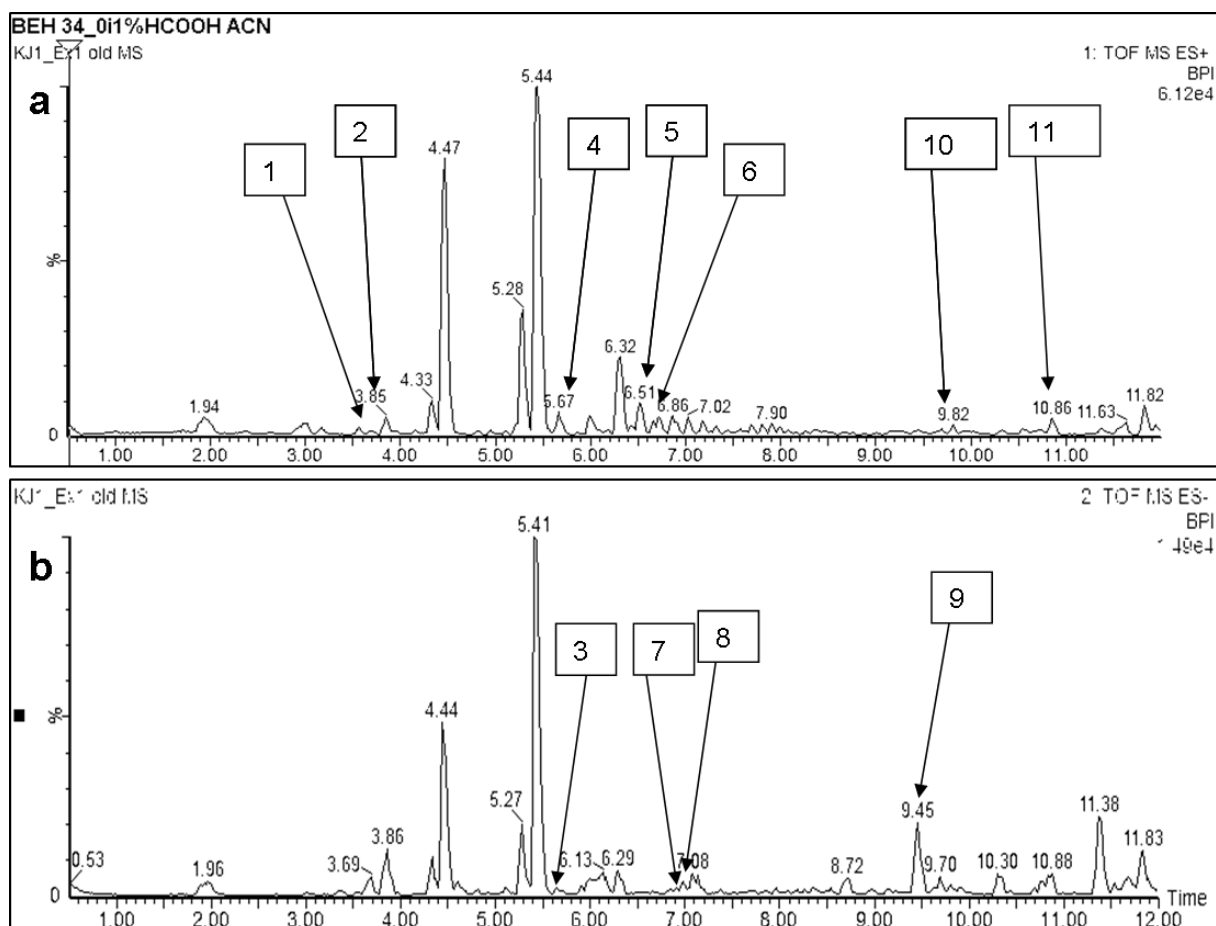


Fig. 2.6 UPLC-TOF-MS chromatograms (base-peak intensity against retention time) obtained in a) ESI+ positive and b) ESI- negative ionization mode of a methanol extract from *H. mantegazzianum* root exudates used for the May 2011 bioassay. The numbers by the peaks indicate retention times. The numbers in boxes indicate compounds that were present only in exudates and not in the control; their molecular weights and tentative molecular formulae are given in Table 4.

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Intraspecific variability in allelopathy of *Heracleum mantegazzianum* is linked to metabolic profile

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Abstract

Allelopathy possibly drives invasions of certain exotic plants. However, there is a lack of studies documenting variability within species in the field of chemically mediated plant-plant interactions. Here, we addressed intraspecific variability in allelopathy of *Heracleum mantegazzianum*, an invasive species with a considerable negative impact on native communities and ecosystems. Variation partitioning among areas, populations within areas and maternal lines within populations sampled in the Czech Republic indicates that the highest variance in allelopathic effects lies among maternal lines. In addition, we performed a comprehensive investigation of the invader's root exudates using UHPLC-TOF-MS-based metabolic profiling and found the metabolic profile to be linked to inhibition of the co-occurring native species *Plantago lanceolata* in a bioassay experiment. Moreover, we tentatively identified 15 compounds associated with this inhibition. We present strong evidence that intraspecific variability should be considered in research on allelopathy and suggest metabolic profiling as an efficient tool for studying chemically mediated plant-plant interactions whenever unknown metabolites are involved.

Introduction

According to the novel weapons hypothesis, invasive exotic species possess novel chemical compounds that have allelopathic effects in new ranges and thus facilitate their invasions (Callaway & Aschehoug 2000; Hierro & Callaway 2003; Callaway & Ridenour 2004). However, many studies failed to prove allelopathy as an important driver of invasions (Blair et al. 2006; Wille et al. 2013; Del Fabbro et al. 2014), which may be caused by methodological difficulties of investigation concerning allelopathy. Firstly, when allelopathy is studied experimentally, the examined plant extract or leachate contains putative allelochemicals, but it also causes changes in pH, osmotic potential, and nutrient concentrations and availability, making it difficult to distinguish allelopathy from resource competition (Inderjit and Callaway 2003). Secondly, laboratory bioassays do not directly describe the ecological complexity. For example, phytotoxicity found *in vitro* is not necessarily applicable under field conditions, because it can be significantly modified by the soil biota (Kaur et al. 2009; Inderjit and van der Putten 2010; Lankau 2010). In addition, effects of bioactive compounds are often concentration-dependent, though the estimation of environmental concentration of allelochemicals is not straightforward, as they are subject to differential adsorption and degradations (Barto and Cipollini 2009).

Another source of variation is likely to be intraspecific variation in the production of allelochemicals. Previous studies have mostly considered allelopathy as a species trait, and when intraspecific differences were tested, they were focused on biogeographical comparisons (e. g. Thorpe et al. 2009). However, within plants, there are different levels of spatial and temporal chemical variation (Macel et al. 2010). As with any trait, allelopathy is influenced not only by evolutionary history, but also by environmental conditions, and therefore quantifying variation and factors influencing this variation is key to a better understanding of plant interactions (Inderjit et al. 2011). Importantly, intraspecific variation in allelopathy can reflect fundamental ecological processes. For example, Lankau & Strauss (2007) showed that competitive interactions between an invader and native species maintain genetic variation in allelopathy of the invader. Similarly, competitive interactions also determined genetic variation in allelopathy of the invader *Alliaria petiolata*. Specifically, Lankau et al. (2009) demonstrated allelopathy strength to be related to invasion history of the invader, with younger populations being more phytotoxic likely due to higher probability of competition with native heterospecifics.

Heracleum mantegazzianum (giant hogweed) is a very well studied invasive plant that has a strongly negative impact on the structure of native communities (Dostál et al. 2013) and the properties of entire ecosystems (Jandová et al. 2014a). Junttila (1976) and Baležentienė (2012) reported invasive conspecifics of *H. mantegazzianum* to be allelopathic; however, Wille et al. (2013) found only limited evidence of its allelopathy on germination of native plants. In a previous study (Jandová et al. 2014b) we found that allelopathic effects of *H. mantegazzianum* exudates in the introduced range varied across several seasons. Apart from possible seasonal variation and despite the origin of all populations sampled for seeds being the Czech Republic, the source of such variability could be considerable intraspecific variation. We suggest that this variation is likely due to differences in the presence and concentration of allelochemical compounds. Since the allelochemicals of *H. mantegazzianum* are unknown, only untargeted metabolomics can reveal these differences.

Metabolomics describes a biological object as the sum of its synthesized metabolites – a metabolome. As such it represents the ultimate response of biological systems to genetic or environmental changes (Fiehn 2002). Recently, untargeted metabolomics has started to be increasingly used in ecology and environmental science. In example, Atkinson et al. (2012) used untargeted metabolomics to test the life history theory when comparing metabolic profiles of storage organs of fast- and slow-growing plants. Davey et al. (2008; 2009) described the effect of a latitudinal climate gradient on the metabolic profile of different populations of *Arabidopsis lyrata* and later tested the effect of cold on their metabolic changes directly in a manipulative experiment. Moreover, untargeted metabolomics has proven to be a good tool for identifying bioactive metabolites in the field of chemical ecology (Nylund et al. 2011; Marti et al. 2013).

Here, we addressed the intraspecific variability of allelopathy in *H. mantegazzianum* using bioassays of its root exudates in combination with metabolic profiling based on ultra-high-performance liquid chromatography (UHPLC) with time-of-flight (TOF) mass spectrometry (MS) detection. Specifically, our aims were i) to estimate the allelopathic effect of root exudates on germination and root development of assay species, ii) to partition the variation in allelopathic effect of *H. mantegazzianum* among areas, populations and maternal lines, and iii) to link the allelopathic effects to metabolic profiles and to tentatively identify compounds responsible for allelopathy.

Methods and materials

Plant material

Seeds of *H. mantegazzianum* were collected from separate maternal plants from 12 populations in six areas. Five out of six areas were in the western part of the Czech Republic, the average distance between these areas was 7 km, and the average distance between populations within these areas was 500 m. One area containing one population was in the central part of the Czech Republic. In each population, we sampled seeds from four randomly selected maternal plants (at a minimum distance of 5 m from each other), reported hereafter as maternal lines (see Fig. 1, S1 for details of the experimental design). All seeds were collected in summer 2012; then they were air-dried and stored in paper bags (seeds from one maternal line in one paper bag) at room temperature. Later on, they were cold stratified in wet sterilised sand at 4°C in the dark for two and a half months, and then let to germinate on wet sterilised sand in the greenhouse with a 20°/10° C temperature regime. The experiment took place at the Institute of Botany, Průhonice, Czech Republic (322 m asl; 49°99'N, 14°57'E). After two weeks of growth, the seedlings were transplanted into 50 mm mesh pots and transferred in randomised order to aeroponic growing systems (Amazon 32, Nutriculture, Lancashire, UK) placed in the same greenhouse and grown in 0.25-strength Hoagland solution for one month. Finally, the plants were collected, and five individuals of one maternal line were placed in one glass bottle with 200 mL of distilled water covered in aluminium foil to keep out the light. The roots were soaked for approximately 20 hours. The water enriched with root exudates was vacuum filtered on glass fibre filters 0.7 µm mesh (Vitrum, Czech Republic), and 4 mL of each sample was taken for a bioassay. The rest was freeze-dried and kept at -20°C until the metabolic profiling using UHPLC-TOF-MS. This procedure yielded on average 35 mg of dry material per maternal line.

Bioassays

Allelopathic effects of root exudates were assayed on commercially purchased seeds of *Arabidopsis thaliana* (Columbia-0 ecotype) and *Plantago lanceolata* (Planta Naturalis, Markvartice, Czech Republic). These species were chosen based on the facts that the former represents standard target species and that the latter represents a species common in grassland communities documented to be invaded by *H. mantegazzianum* (Dostál et al. 2013). Distilled water was used as the control. Petri dishes (6 cm diameter) were fitted with qualitative filter paper (Papírna Perštejn, Czech Republic), ten seeds of an assay species per dish were placed in, 4 mL of root exudate or the control was pipetted in and the Petri dishes were placed in a

chamber with a 25°/10° C temperature and 12/12 h light regime. Bioassays started immediately after the exudates were collected. After 7 days we recorded number of germinated seeds of *A. thaliana*. For *P. lanceolata*, we recorded the number of germinated seeds and the root length of three largest seedlings, both scored after 10 days since the experiment was initiated. We used 5 replicates per maternal line in the bioassay.

Bioassay data analyses

Initially, we used a within-study meta-analytical approach to compare allelopathic effects among maternal lines of *H. mantegazzianum*. We used log-response ratios as effect sizes. Specifically, it was calculated as the natural logarithm of the mean bioassay response to respective exudate divided by the mean bioassay response to the control. Thus, negative values of log-response ratios indicate that the maternal line is allelopathic whereas positive values indicate that such maternal line stimulates germination or root growth of the assay species. The log-response ratios were calculated for *A. thaliana* germination, *P. lanceolata* germination and *P. lanceolata* root length separately using the ‘escalc’ function in the ‘metafor’ package in R (Viechtbauer 2010). Overall effect sizes were analysed with random models to estimate the overall allelopathic effect. In addition, mixed-effects models with the exudates’ dry weights as moderator were fitted to estimate the allelopathic effect independent on the amount of root exudate produced. Then we carried out a variance component analysis to partition the overall variation in allelopathic effects of *H. mantegazzianum* among areas, populations within areas, maternal lines within populations and experimental variation using the ‘lme’ function in the ‘nlme’ package in R (Pinheiro et al. 2014). Root length was log-transformed before variance component analyses. All analyses were performed in R 3.0.2 (R Development Core Team 2013).

Metabolite extraction and UHPLC-TOF-MS-based metabolic profiling

The extraction procedure proposed by De Vos et al. (2007) for untargeted metabolomics studies was followed, except for different extraction ratios. Before metabolite extraction, the freeze-dried root exudates were weighted to allow for later concentration correction. Then, 2.4 mL of chilled 50% methanol, 0.1% formic acid in water was added. After immediate vortexing, the extracts were sonicated for 15 min at room temperature and then filtered through 0.2 µm nylon filters (Costar, USA) in a centrifuge (10 min, 5000 rpm). The extracts were prepared freshly at the beginning of each analysis.

The samples were analysed on an Acquity UPLC system with an LCT premier XE time-of-flight mass spectrometer (Waters) using the LC Column Acquity UPLC BEH C18 (50 mm × 2.1 mm I.D., particle size 1.7 µm, Waters) and a two-component mobile phase. The mobile phase A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The analyses were performed under a linear gradient programme (min/%B) 0/5, 1.5/5, 15/70, 21/95, 23/98 followed by a 1 min equilibration (5% B). The total analysis time was 25 min. The column temperature was set at 40 °C, flow rate at 0.4 mL min⁻¹ and the injection volume was 5 µL. The mass spectrometer operated in the “W” mode with capillary voltage set at +2800 (positive ionisation mode) or -2500 V (negative ionisation mode), cone voltage +40 or -40 V, desolvation gas temperature, 350 °C; ion source block temperature, 120 °C; cone gas flow, 50 L h⁻¹; desolvation gas flow, 800 L h⁻¹; ion guide 1 and 2 RFs, 200 and 300 V, respectively; hexapole RF, 150 V. Ions in the *m/z* range 100–1500 were detected using a scan time of 0.1 s and an inter-scan delay of 0.01 s. The mass accuracy was kept below 5 ppm using lock spray technology with leucine enkephalin as the reference compound (2 ng µl⁻¹, 5 µl min⁻¹). Fragmentation by collision-induced dissociation (CID) was triggered by setting the aperture 1 value at 30 – 50 V. CID fragmentation spectra were recorded under chromatographic conditions described earlier (system 1) and using the same chromatographic system modified by replacing acetonitrile for methanol as part B of the mobile phase (system 2). Since no significant differences among triplicates of initially analysed samples were observed, only one replicate per sample was measured. Samples were run in a random order in a single batch and MS detection was performed in both negative and positive modes in independent runs. The data were processed by MassLynx V4.1 (Waters). To confirm the compound identification, we compared retention times and fragmentation patterns with the commercially available standards of bergapten (Aldrich), xanthotoxin (Fluka) and tryptophan (Sigma-Aldrich), which were dissolved in methanol and analysed using the same protocol as described above.

Metabolomic data processing

LC-MS data were automatically processed by MetAlign version 3 (Lommen and Kools 2012). Baseline and noise calculations were performed from scan number 10 to 3500 corresponding to retention times of 1.0 to 16.0 min. The maximum amplitude was set to 40000, and peaks below twice the local noise were discarded. For subsequent data analyses, the data matrices (retention time × accurate *m/z* × peak intensity) from positive and negative ionisation modes were used separately. The matrices were reduced by discarding variables with maximum

signal below the intensity of 100. All data matrices were then normalised to total signal intensity by observations and then log-transformed and centred by variables. Orthogonal partial least squares analysis (OPLS) was applied to test whether allelopathy of exudates can be explained by their metabolic profile and to mine out metabolomic peaks (characterised by retention time $\times$ accurate m/z) most related to the magnitude of the allelopathic effect in the bioassay from significant models. The OPLS method is designed to separate the predictive part of the data related to the y-variable from variation that is not related to the response (Trygg and Wold 2002). Here, we used effect sizes of responses in the bioassay as y-variables. OPLS CV-ANOVA (Eriksson et al. 2008) was used to test the significance of each model. To indicate compounds associated with allelopathy, loading column plots were produced, and peaks (characterised by retention time $\times$ accurate m/z) most related to the magnitude of the allelopathic effect were extracted. Further, the models were examined to identify possible influential outliers using Hotelling's T^2 , which is the sum over all components of scores squared divided by their squared standard deviation. All multivariate data analyses were performed using SIMCA-P software (Umetrics AB, Umeå, Sweden). In addition, we tentatively identified compounds corresponding to extracted metabolomic peaks by processing the LC-MS data. Elemental compositions were proposed based on accurate mass and isotopic pattern recognition using MassLynx V4.1 (Waters). The resulting molecular formulae were submitted to the Reaxys and SciFinder databases. The proposed molecular structures were then confronted with our MS fragmentation experiments.

Results

i) An overview of allelopathic effects

The effect of root exudates was species-specific. *A. thaliana* was stimulated by *Heracleum mantegazzianum* root exudates (Table 1, Fig. 2A). Conversely, germination and root length of *P. lanceolata* were significantly inhibited by the root exudates of *Heracleum mantegazzianum* (Table 1, Fig. 2B, 2C). We observed substantial differences between maternal lines that could be attributed to both qualitative and quantitative properties of root exudates. Nevertheless, the maternal lines that suppressed most the germination also suppressed root length ($t = 4.1891$, $df = 39$, $p\text{-value} < 0.001$, $r = 0.557$). To examine qualitative attributes only, we analysed the effect sizes together with dry weight of exudates used as a moderator. When the amount of root exudate produced was incorporated in the analyses, we failed to find any effect of exudates on germination of *A. thaliana* (Table 1). The exudate concentration did not modify the effect on

P. lanceolata germination; however, the effect on root length of *P. lanceolata* was significantly negatively dependent on the dry weight of root exudates, and as a result, incorporation of the moderator inverted the final effect size to positive values (Table 1). Our results therefore suggest that allelopathic effects are underlined by both qualitative and quantitative properties of root exudates.

ii) Partitioning of variation in allelopathic effects of *Heracleum mantegazzianum* among areas, populations and maternal lines

Variance partitioning showed that the highest biological variation in allelopathic effects lies in variation among maternal lines. There were 15.6% and 38.0% of the total variance in the germination and root length of *P. lanceolata*, respectively, attributed to allelopathic differences among maternal lines. Differences in allelopathy among populations or areas had a negligible contribution to variation in the response variables (Table 2). However, most of the differences in germination and root length of *P. lanceolata* were due to high variability among experimental replicates, with 84.4% and 60.1% of the total variance, respectively. An alternative analysis was performed that took into account only two areas in which our sampling design was perfectly balanced, with three populations within each area and four maternal lines within each population. This did not change the results (presented in Table S1) in any substantial way.

iii) Linkage of allelopathic effects to metabolic profiles

Orthogonal partial least squares analysis (OPLS) was applied to model the respective bioassay response by the metabolic profile and to indicate peaks associated with allelopathy. We modelled both ionisation modes and each bioassay response separately, resulting in 6 particular models described in Table 3a. Statistically significant models were never obtained to explain the effect of the metabolic profiles on *A. thaliana* germination. By contrast, the germination and root length of *P. lanceolata* were always significantly explained by the metabolic profile in OPLS (P-value < 0.05). Metabolomic peaks most related to the magnitude of the allelopathic effect in the bioassay were extracted from significant models (Fig. 3). We found one maternal line with significantly higher influence (P-value < 0.01) on our models (Fig. S2) according to Hotelling's T². When this maternal line was excluded from the OPLS analyses, only the model explaining root length of *P. lanceolata* by the metabolic profile measured in negative ionisation mode remained significant (Table 3b). We extracted

peaks most related to the allelopathic effect from the latter model to check whether they lead to the same compounds as the models based on all observations did (Fig. S3).

iv) Characterisation of compounds associated with allelopathy

We subsequently examined the peaks most related to the magnitude of allelopathic effect and the associated UHPLC-TOF-MS data, which led us to 15 individual compounds summarized in Table 4. Several compounds were identified in the OPLS models by more than one peak. Interestingly, 14 out of 15 compounds can be categorized into only three groups according to their chemical structure: (1) two dipeptides and one amino acid, (2) six C18 oxylipins, and (3) five malonyl-monoglycosides. It should also be noted that several compounds, each of them identified by at least one peak, are chromatographically separable isomers of identical elemental composition – e.g. compounds IV, V and VI listed on a common row in Table 4. Moreover, peaks extracted from OPLS modelling of either bioassay response mostly lead to the same compounds, suggesting that both germination and root length of *P. lanceolata* are affected by the same compounds.

We carried out CID MS fragmentation experiments to obtain a more detailed insight into the compound structures. MS data for compounds I and II correspond to dipeptides., Compound I is tentatively composed of common yet non-proteinogenic aminoacids: hydroxyleucine or hydroxyisoleucine and hydroxyproline, as depicted in Fig. 4A. We have not found such dipeptide, which complies with both the elemental composition and fragmentation data in the databases. Compound II was tentatively identified as leucyl-proline or isoleucyl-proline (Fig. 4B). Compound III was identified as tryptophan based on elemental composition and comparison of retention times and fragmentation patterns in the CID MS spectra with those of the authentic standard (Fig. 4C).

Compounds IV - VI are C18 fatty acids according to their elemental composition and corresponding hits in the databases. This assumption was supported by the presence of $[M-H-CO_2]^-$ fragments in the negative ionisation CID MS spectra, confirming the presence of a carboxyl group in the structures. Further, all the structures contain at least one additional oxygen besides those of the carboxyl group, which specifies these compounds as C18 oxylipins. Unfortunately, the MS data did not allow us to localise double bonds and/or oxygen-containing functionalities in the structures. Nevertheless, several compounds that correspond to the MS data were found in the inquired databases. One of them, corresponding to the compound VIII, is 12-oxo-phytodienoic acid (Fig. 4D), an important precursor of a plant hormone, which we discuss below. The other oxylipins we found in root exudates of

Heracleum mantegazzianum may represent derivatives of 12-oxo-phytodienoic acid or a similar compound.

Compounds X - XIV are malonyl-monoglycosides, all of them composing of a malonyl moiety attached to a saccharide unit, which is further ornamented with an aliphatic or alicyclic side chain as shown in Fig. 4E. The MS data do not enable to determine type of the saccharide nor exact structure of the side-chain. However, for the isomers X - XII and for isomers XII-XIV, the inquired databases revealed specific compounds of the respective elemental compositions, which contain a malonyl-monosaccharide moiety. Namely, it was 1-geranyl-6-O-malonyl- β -D-glucopyranoside (GMGP) and 1-linaloyl-6-O-malonyl- β -D-glucopyranoside (LMGP), Fig. 4F, corresponding to the isomers X - XII and monardin F and 1'-ethenyl-5'-hydroxy-1',5'-dimethyl-3'-hexen-1-yl-6-O-malonyl- β -D-glucopyranoside, Fig. 4G, corresponding to the isomers XIII - XIV. Except for monardin F, the compounds contain a glucose unit bearing an aliphatic side-chain. Interestingly, monardin F shows different properties in its molecule because it is composed of an allose unit and an alicyclic side-chain. We propose that all compounds X - XIV contain the same saccharide moiety; it, however remains unclear whether it is glucose, which we consider more likely, or allose or any other saccharide moiety. It is even more difficult to predict the exact structure of the side-chain, which can be either aliphatic or alicyclic. In addition, we assume that the isomeric compounds differ in the position of a double bond and/or hydroxyl group.

Concerning compound XV, we were unable to reliably identify even its pseudomolecular ion, therefore; more detailed information on this substance is not available.

The insufficient amount of root exudate extracts did not enable purification of the compounds for nuclear magnetic resonance analysis in order to further elucidate the compound structures.

In addition, we searched for furanocoumarins, which are typical secondary metabolites in *Heracleum mantegazzianum* (Tiley et al. 1996) and were previously reported to be responsible for allelopathic effect (Macías et al. 1993; García et al. 2002). We found two furanocoumarins, xanthotoxin and bergapten, present in the exudates according to elemental composition and comparison of retention times and fragmentation patterns in the CID MS spectra with those of authentic standards. However, these were not connected to the magnitude of the allelopathic effect.

Discussions and conclusions

Allelopathy may drive the invasive success of exotic species (Callaway & Aschehoug 2000; Hierro & Callaway 2003; Callaway & Ridenour 2004). Most research carried out so far has focused on species-level patterns in allelopathy, and little is known about intraspecific variation of allelopathy within plants (but see Lankau et al. 2009). Here we provide strong evidence that one of the worst invaders in Europe (DAISIE European Invasive Alien Species Gateway 2008), *H. mantegazzianum* is phytotoxic towards native plant *P. lanceolata*, although the effect is subject to considerable variation between different maternal lines. Generally, the phytotoxicity of root exudates against *P. lanceolata* is dependent on both the quantitative (amount) and qualitative (composition) properties of the exudate produced. Apart from intraspecific variability in the production and effects of allelopathic compounds, our findings showed allelopathy in *H. mantegazzianum* to be species-specific in terms of perception by other species.

Biological variation in allelopathic effects was highest at the maternal lines level and very low between different areas or populations in which we sampled seeds. The lack of spatial structure in allelopathic effects among populations and even areas has already been observed and may be due to growing plants under common conditions (Kunin et al. 2009). Had the differences in allelopathic effects resulted from environmental conditions such as the temperature or water regime, we would have observed much higher total variability and a stronger spatial pattern in plants sampled directly in the field. Here, we can neither assess the sources of intraspecific variation, nor tell the reason why this variation is sustained in our system. We are also lacking information on the costs of allelopathy in *H. mantegazzianum* as shown on the example of *Brassica nigra* (Lankau and Strauss 2007; Lankau 2008) or whether the species itself is negatively affected by its own root exudates as shown for other species (Canals et al. 2005; Alías et al. 2006), being the mechanisms that could select for a decrease in allelopathic potential. Specifically, Lankau (2008) found an investment in allelochemicals in *Brassica nigra* to be favoured when the plant competed mostly with heterospecifics; it, however, negatively correlated with competitive ability towards conspecifics. On the other hand, the invasive *Brassica rapa* and *Centaurea maculosa* invested in secondary metabolites to maintain chemical defence without any decrease in growth rate or similar competitive traits (Siemens et al. 2002; Ridenour et al. 2008; respectively). Trade-offs related to the production of allelochemicals in *H. mantegazzianum* need to be estimated in order to explain why the variability is sustained in our system.

Untargeted metabolic profiling allowed us to investigate the relationship between allelopathy and the metabolic profile without prior knowledge of the allelopathic chemicals. The variation in allelopathic effects on *P. lanceolata* germination and root length among different maternal lines turned out to be determined by metabolic profiles of respective root exudates gained by UHPLC-TOF-MS. However, one maternal line had strong influence on our models, and its exclusion led to loss of significance in all but the model explaining root length of *P. lanceolata* by the metabolic profile measured in negative ionisation mode. The weak predictive power could have resulted from the fact that the predictive component represented only about one third of the systematic variation. We suggest that there was another important source of metabolic variation in our maternal lines apart from allelopathic effect. Such metabolic variation could be related, for example, to chemical defence against herbivores or pathogens, since it has been shown not to relate to allelochemicals in other species (Uesugi and Kessler 2013). Nonetheless, we tentatively identified 15 compounds associated with allelopathic effects based on models containing all observations and further confirmed three out of six C18 oxylipins (compounds IV – VI) and all the malonyl-glycosides (compounds X – XIV) when we based our conclusions only on the model which remained significant after the exclusion of the outlying maternal line. Compounds of particular interest are discussed below.

Plant oxylipins are a structurally and functionally very diverse group of compounds derived from the oxidation of polyunsaturated fatty acids. Plant oxylipins were shown to be involved in signalling and defence mechanisms against various herbivores or pathogens (Mosblech et al. 2009). 12-oxo-phytodienoic acid is among the best known representatives of this group. It is a precursor of the plant hormone jasmonic acid. Apart from being a precursor, the compound itself and its derivatives have been reported to be effective regulators of defence responses (Blée 2002). To the best of our knowledge, there is only one study on phytotoxicity of plant derived oxylipins; Fiorentino et al. (2008) found several hydroxylated unsaturated C18 oxylipins in extracts from the Mediterranean shrub *Cestrum parqui* that inhibited the germination and growth of *Lactuca sativa*, and their bioactivity depended on the number of double bonds present in the molecules.

The isomers X - XII represent 6-O-malonyl-glycosides of certain monoterpenols. These compounds were not previously reported in plants from the *Apiaceae* family, although they have been found in different fruits and vine leaves (Withopf et al. 1997), whole plants of *Monarda punctata* (Yamada et al. 2010), flower buds of *Jasminum sambac* (Moon et al. 1994), and in flowers of *Boronia megastigma* (Cooper et al. 2011). Isomers XIII, XIV

represent 6-O-malonyl-glycosides of some monoterpenoids that are likely hydroxyderivatives of isomers X - XII and that were previously described in *Boronia megastigma* (Cooper et al. 2011). In general, terpenoid glycosides are regarded as transport and storage forms of terpenoids in plant tissues, and they have been recognized to play important roles as precursors of aglycone release (Winterhalter and Skouroumounis 1997). Volatile terpenoids play many ecological roles in plant chemical defences and communication (Gershenzon and Dudareva 2007). Importantly, Yang et al. (2013) found compounds tentatively identified as linalool-malonyl-glucoses in leaves of *Chrysanthemum morifolium* where they functioned as herbivore insect deterrents. Their study therefore suggested for the first time that linalool glycosides play a biological role other than storage of volatiles. We can hypothesize that monoterpenoid 6-O-malonyl-glycosides we found in root exudates would function also as allelochemicals; however, direct proof of this effect will require purification of these compounds and direct bioassays.

We also found two furanocoumarins, xanthotoxin and bergapten, present in the exudates, however, these were not connected to the magnitude of the allelopathic effect. This contradicts previous studies on *Pilocarpus goudotianus* leaves (Macías et al. 1993) or on seeds of *Ammi majus* (García et al. 2002) where furanocoumarines were responsible for allelopathy of these species. In general, phenolics are considered to be responsible for allelopathy in many plants (Inderjit 1996; Kim & Lee 2010; Li et al. 2010), however, here we indicated compounds belonging to different structural groups to be associated with allelopathy.

This study presents results of a comprehensive investigation of the metabolic profile of root exudates of the infamous invasive species *H. mantegazzianum* together with bioassays testing the effects of root exudates on germination and root development of other native species. We uncovered strong evidence that investigating variability in allelopathy should be included in studies of the novel weapons hypothesis. In addition, we employed state-of-the-art techniques in allelopathy research and suggest that environmental metabolomics is an efficient tool for studying plant-plant interactions whenever unknown metabolites are involved or already described metabolites fail to explain the full effect observed in nature.

Table 3.1 Overall effect sizes estimated with random-effects models and effect sizes with dry weight of root exudates used as a moderator estimated with mixed-effects models, both by the restricted maximum-likelihood method. Shown are degrees of freedom (df) as well as model estimates of effect sizes with their standard errors (SE) and Z-values and levels of significance (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$) for each response measured in the bioassay.

	df	Estimate	SE	Z-value	
Without moderator					
Germination of <i>A. thaliana</i>	40	0.0601	0.0255	2.3618	*
Germination of <i>P. lanceolata</i>	40	-0.5509	0.0410	-13.4402	***
Root length of <i>P. lanceolata</i>	40	-0.2415	0.0736	-3.2803	**
With dry weight of exudates used as a moderator					
Germination of <i>A. thaliana</i>					
Intercept	39	0.1174	0.0658	1.7823	NS
Exudate dry weight	1	-1.4318	1.519	-0.9426	NS
Germination of <i>P. lanceolata</i>					
Intercept	39	-0.4444	0.1028	-4.3245	***
Exudate dry weight	1	-2.7711	2.4568	-1.1279	NS
Root length of <i>P. lanceolata</i>					
Intercept	39	0.3715	0.1598	2.3255	*
Exudate dry weight	1	-15.4328	3.7478	-4.1178	***

Table 3.2 Proportions of total variance (%) in allelopathic effects of *H. mantegazzianum* attributed to variation among areas, among populations within areas, among individual maternal lines within populations and experimental variation.

	Among areas	Among populations	Within populations	Residuals
Germination of <i>P. lanceolata</i>	<0.0001	<0.0001	15.6	84.4
Root length of <i>P. lanceolata</i>	1.9	<0.0001	38.0	60.1

Table 3.3 Orthogonal partial least squares (OPLS) models applied to model the respective bioassay response (y-variable) by the metabolic profile. Each electrospray ionisation mode (positive, ESI+ and negative, ESI-) and each bioassay response were modelled separately. Log-response ratios were used as effect sizes. Models with all observations (**a**) and model that remained significant after exclusion of outlying maternal line (**b**). Shown are: N, number of observations; K, number of variables; R2X(cum), fraction of variation of metabolomic data explained by the model; R2X(pred), fraction of systematic variation in the metabolomic data related to the y-variable; R2X(ortho), fraction of systematic variation in the metabolomic data non-related to the y-variable; R2Y, fraction of variation of the y-variables explained by the model; Q2Y, total variation of the y-variable predicted by the model. *F*-values (*F*) and levels of significance (*P*) were computed by CV-ANOVA.

ESI	y-variable	N	K	R2X(cum)	R2X(pred)	R2X(ortho)	R2Y	Q2Y	<i>F</i>	<i>P</i>
(a)										
ESI+	Effect sizes of <i>A. thaliana</i> germination	41	2248	0.319	0.035	0.884	0.195	0.008	0.048	0.999
ESI+	Effect sizes of <i>P. lanceolata</i> germination	41	2248	0.291	0.091	0.200	0.736	0.297	3.795	0.011
ESI+	Effect sizes of <i>P. lanceolata</i> root length	41	2248	0.298	0.108	0.189	0.769	0.494	8.794	<0.001
ESI-	Effect sizes of <i>A. thaliana</i> germination	41	3649	0.296	0.0413	0.255	0.903	0.187	1.304	0.282
ESI-	Effect sizes of <i>P. lanceolata</i> germination	41	3649	0.265	0.094	0.171	0.751	0.298	3.825	0.011
ESI-	Effect sizes of <i>P. lanceolata</i> root length	41	3649	0.269	0.121	0.148	0.807	0.491	8.686	<0.001
(b)										
ESI-	Effect sizes of <i>P. lanceolata</i> root length	40	3649	0.332	0.078	0.254	0.989	0.526	4.297	0.002

Table 3.4 Compounds associated with allelopathy of *H. mantegazzianum* revealed in the bioassay with *P. lanceolata*. Peaks extracted from OPLS models based on all observations are presented in bold. Bioactivity shows whether the peaks were identified based on inhibition of *P. lanceolata* germination (g) or root length (r). In addition, compounds indicated based only on the model explaining root length of *P. lanceolata* by metabolic profile measured in negative ionisation mode that remained significant after the exclusion of the outlier are presented in bold. ESI stands for electrospray ionisation mode; positive (ESI+), negative (ESI-).

Compound number*	Retention time (min)	Molecular formula	Adduct/fragment identified in MS spectrum		Bioactivity	Tentative identification**	
	in system 1: acetonitrile mobile phase		ESI+	ESI-		DBE - double bond equivalent O - nr of oxygens besides carboxyl group	Compound category
I	1.15	C11H20N2O5	261.1450 [M+H]⁺	259.1302 [M-H] ⁻	g, r		Aminoacids/ Dipeptides
II	2.04	C11H20N2O3	229.1544 [M+H]⁺	227.1375 [M-H] ⁻	g		
II	1.46	C11H12N2O2	188.0807 [M-NH₃+H]⁺	249.076 [M+HCOOH-H] ⁻	g	tryptophan	
			205.0969 [M+H] ⁺				
IV, V, VI	12.63; 12.90; 13.13	C18H30O3	277.2147 [M-H₂O+H]⁺	293.2117 [M-H]⁻	g, r	DBE 4	C18 oxylipins
			295.2282 [M+H]⁺	339.2154 [M+HCOOH-H]⁻		O 1	
			296.2315 A+1 isotope of [M+H]⁺				
			317.2075 [M+Na]⁺				
			333.1820 [M+K] ⁺				
			358.2338 [M+Na+CH ₃ CN] ⁺				
VII	10.5	C18H34O4	279.2305 [M-2H ₂ O+H] ⁺	313.2353 [M-H] ⁻	g, r	DBE 2	
			297.2412 [M-H ₂ O+H] ⁺			O 2	
			315.2519 [M+H]⁺				
			337.2339 [M+Na] ⁺				
			353.2079 [M+K] ⁺				
VIII	12.04	C18H28O3	293.2099 [M+H]⁺	291.1954 [M-H] ⁻	g, r	DBE 5	
			315.1921 [M+Na] ⁺	337.2010 [M+HCOOH-H] ⁻		O 1	
IX	12.38	C18H32O3	279.2333 [M-H₂O+H]⁺	295.2268 [M-H] ⁻	g, r	DBE 3; O 1	

X, XI, XII	7.23; 7.41; 7.01	C19H30O9	420.2209 [M+NH ₄] ⁺	403.1967 [M+H ₂ -H] ⁻	g, r	R-O-Malonyl-O-monoglycoside	Malonyl-monoglycosides
			425.1761 [M+Na] ⁺	404.2014 A+1 isotope of [M+H ₂ -H] ⁻		R = C10H17	
			441.1495 [M+K] ⁺	803.3701 [2M-H] ⁻			
				804.3745 A+1 isotope of [2M-H] ⁻			
				357.1907[M-CO ₂ -H] ⁻			
XIII, XIV	4.64; 4.75	C19H30O10	401.1816 [M+H] ⁺	419.1939 [M+H ₂ -H] ⁻	g, r	R-O-Malonyl-O-monoglycoside	
			441.1690 [M+Na] ⁺	420.1950 A+1 isotope of [M+H ₂ -H] ⁻		R=C10H17O	
			457.1441 [M+K] ⁺	835.3622 [2M-H] ⁻			
				836.3633 A+1 isotope of [2M-H] ⁻			
XV	4.78	(C39H60O18)***	N.D.	815.3700 [M-H] ⁻	g	Unknown	

* Isomers of the same molecular formula, but differing in their retention times are listed in one common row

** Based on LC-MS data including CID fragmentation

*** The identity of the pseudomolecular ion was not confirmed by the presence of an adduct or fragment; the prediction of elemental composition cannot therefore be considered reliable

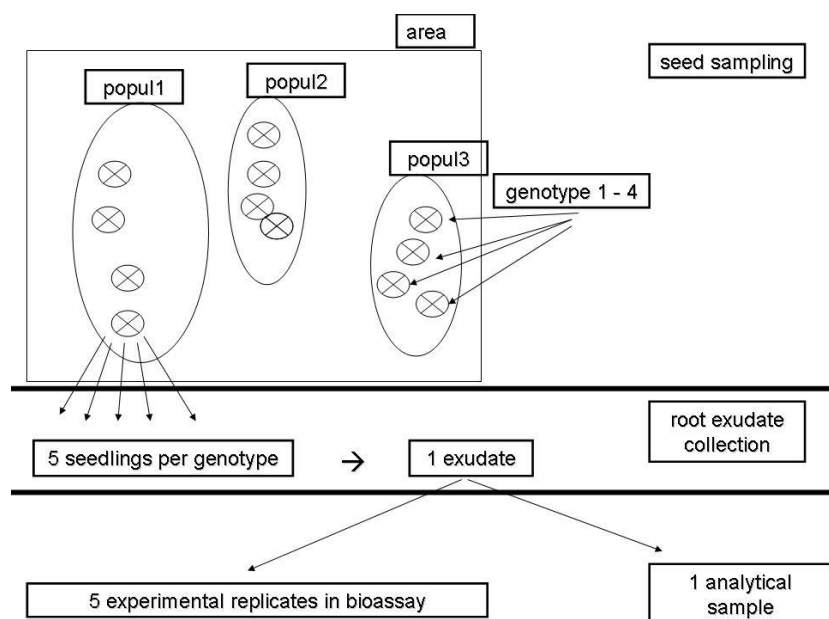
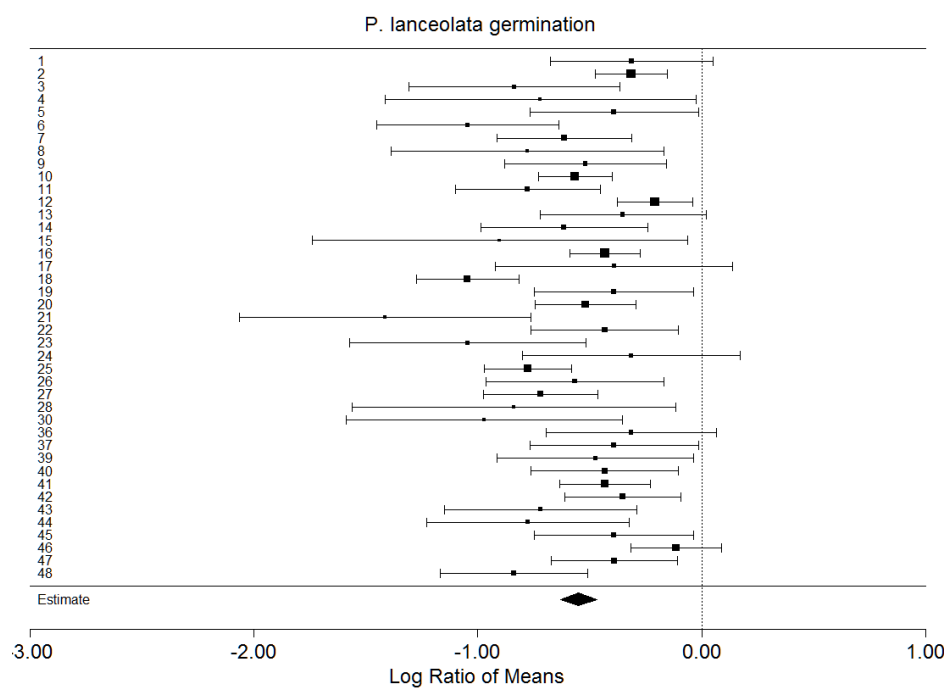
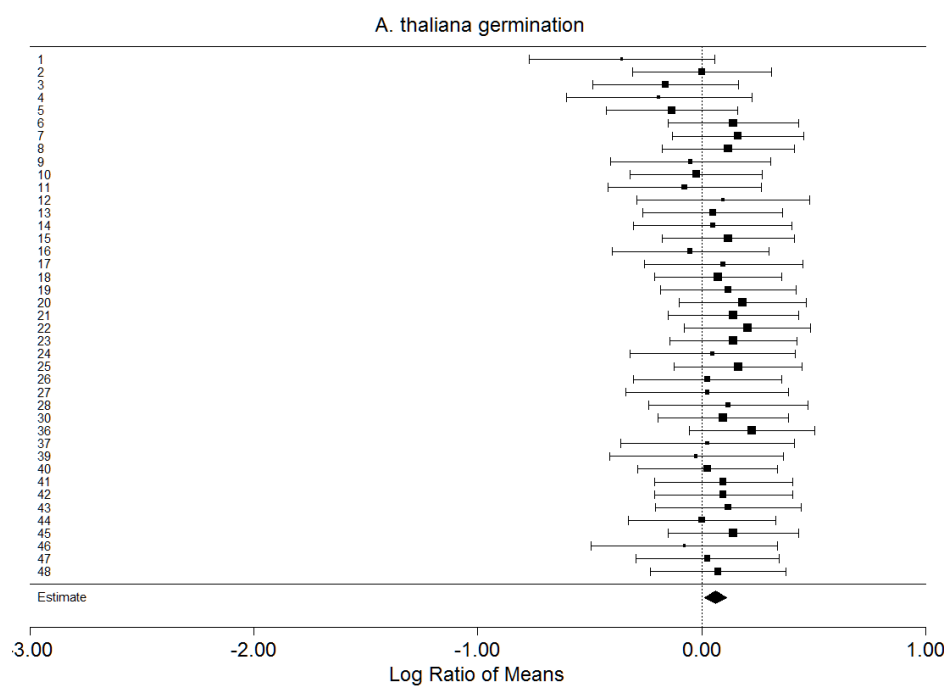


Fig. 3.1 Experimental design of the study.



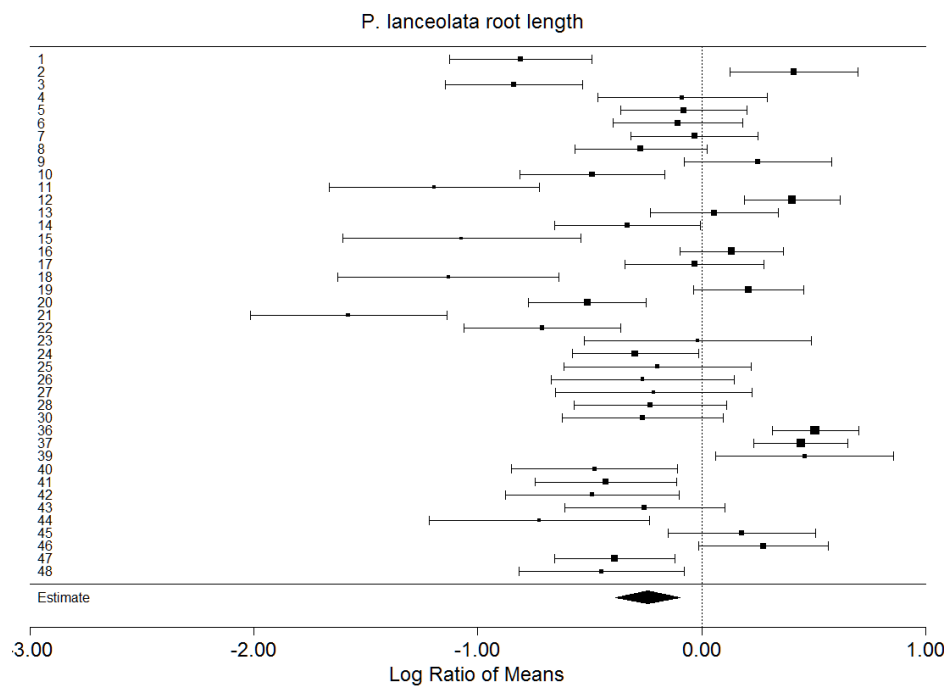
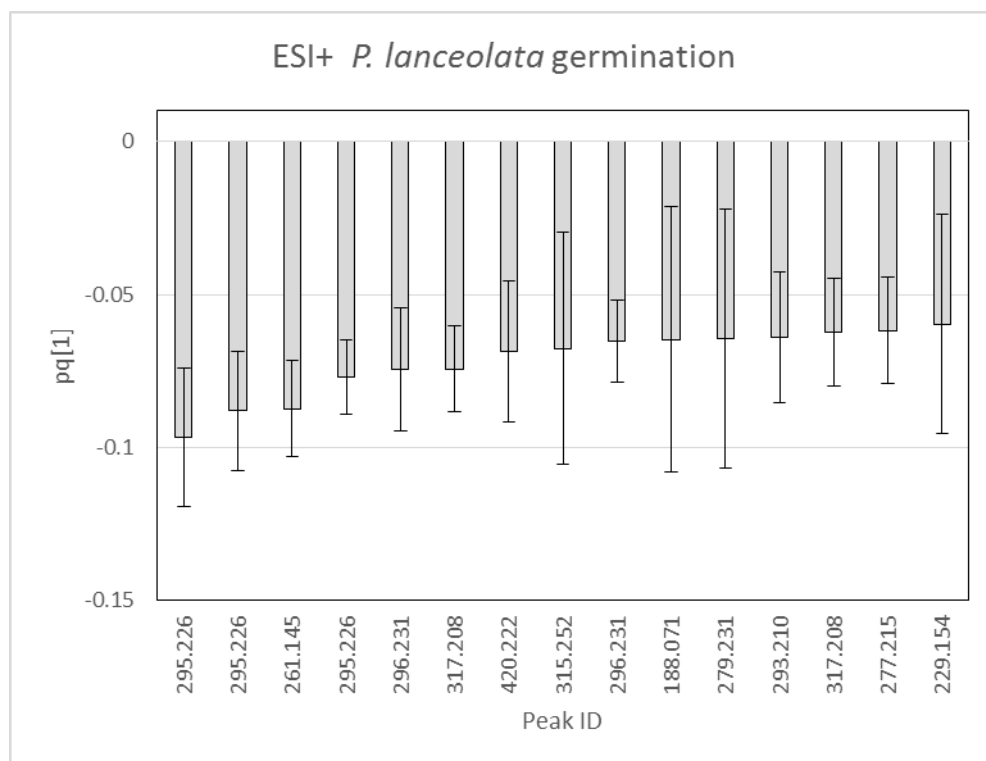
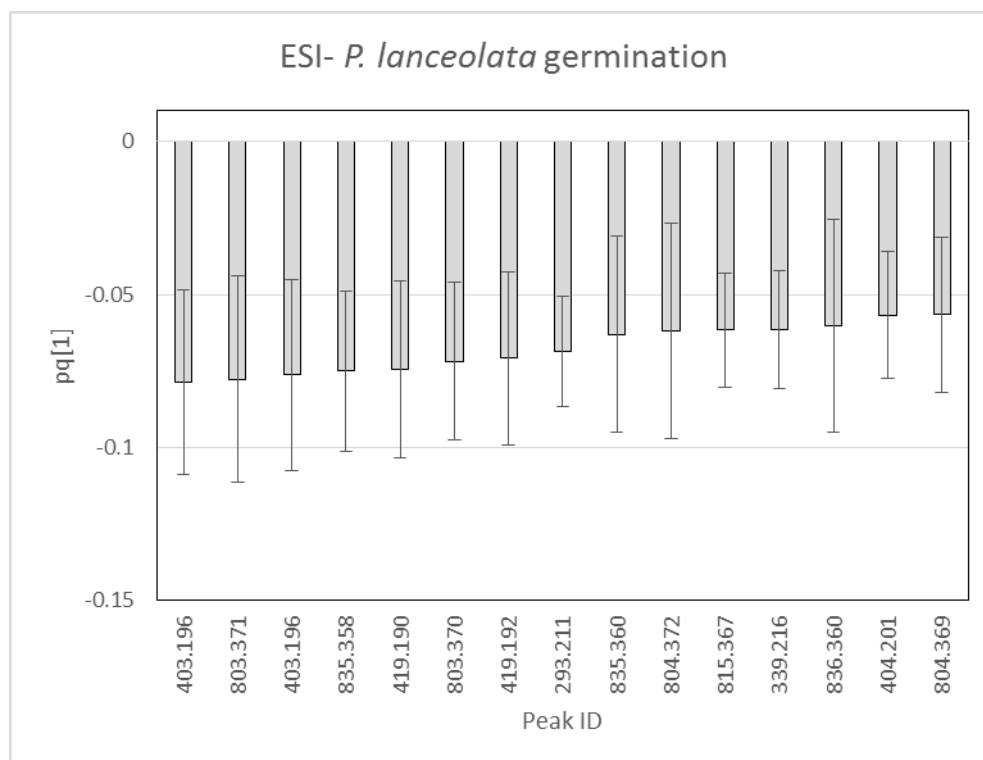
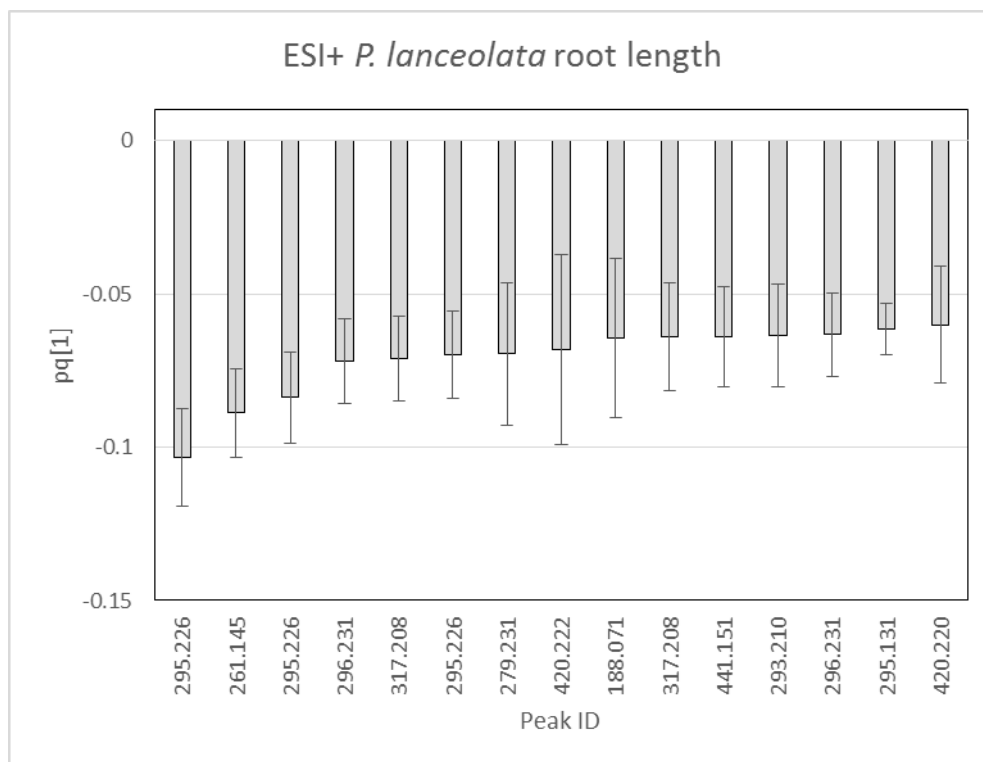


Fig. 3.2 Overview of allelopathic effects. Effect sizes of root exudates of different maternal lines of *Heracleum mantegazzianum* on germination of *A. thaliana* (**A**) and germination and root length of *P. lanceolata* (**B** and **C**; respectively). The figures show the log-response ratios with corresponding 95% confidence intervals in the individual maternal lines and the estimates of overall effect size based on random-effects models fitted by the restricted maximum-likelihood method.





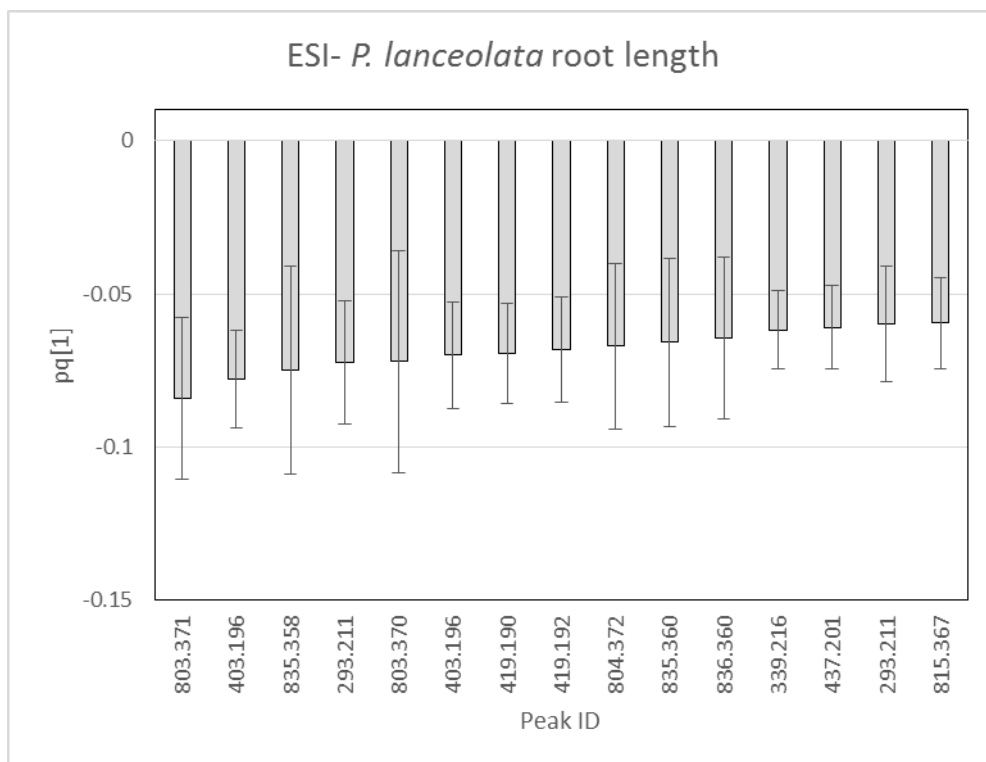
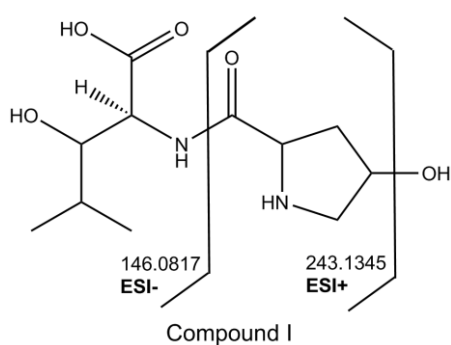
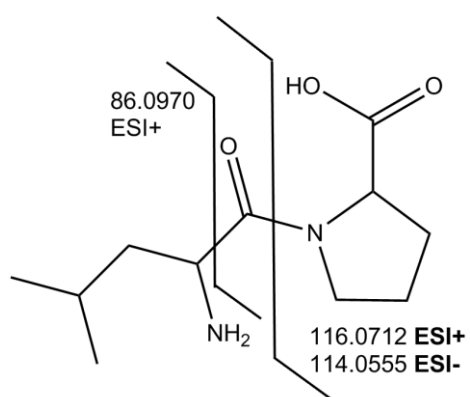


Fig. 3.3 Loading column plots of orthogonal partial least-square (OPLS) models. The models are described in Table 3. The plots show metabolomic peaks (characterised by retention time $\times$ accurate m/z) most associated with allelopathic effects that were extracted from the analyses of full metabolomic data. On the x-axes, the peaks are represented by their m/z values. The y-axes represent the predictive components of the OPLS models. Negative values of predictive components are associated with negative values of effect sizes in the bioassay, *i.e.* allelopathy. The error bars were calculated by jack-knifing (Efron and Gong 1983) in SIMCA-P software.

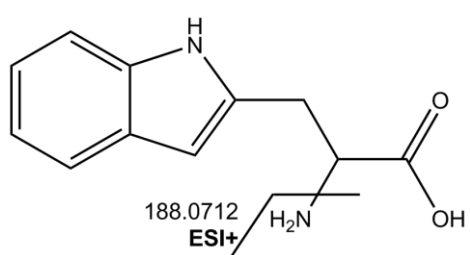


4A



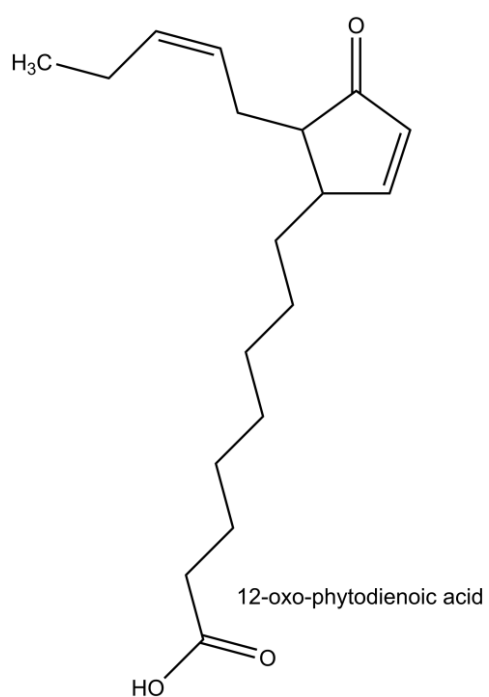
Compound II

4B

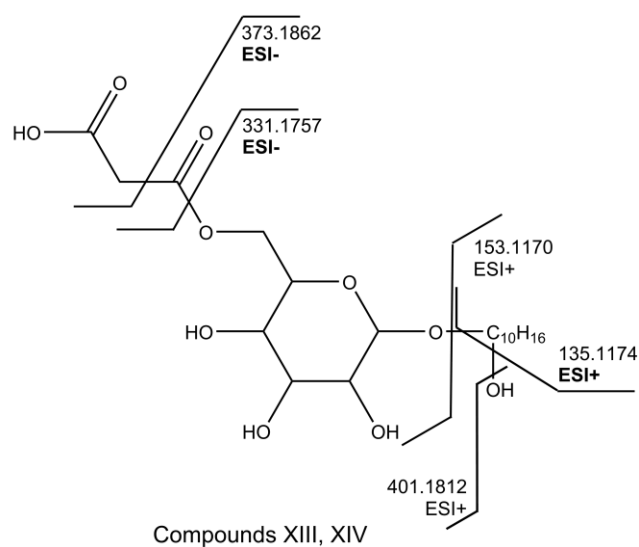
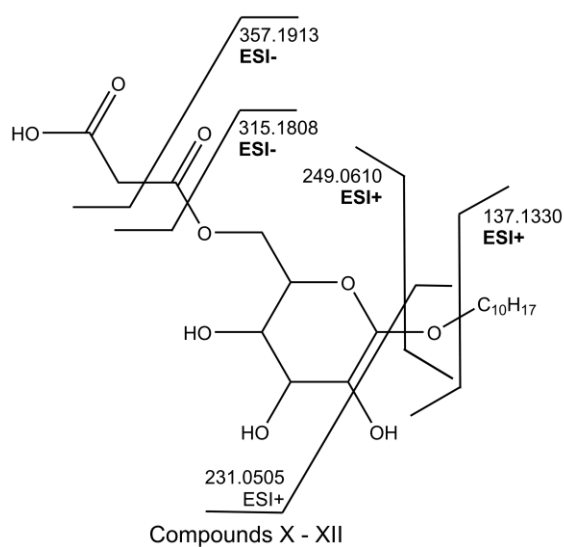


Compound III
Tryptophan

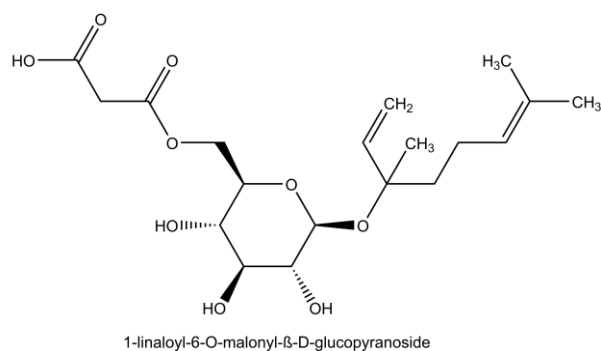
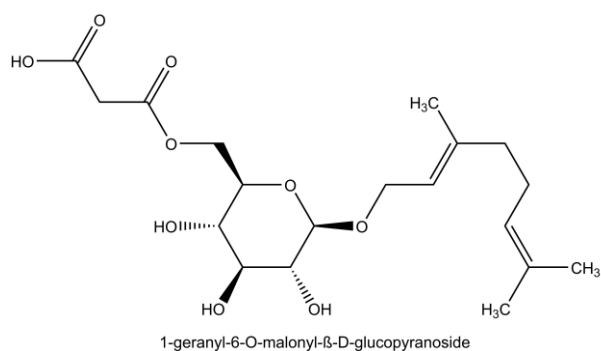
4C



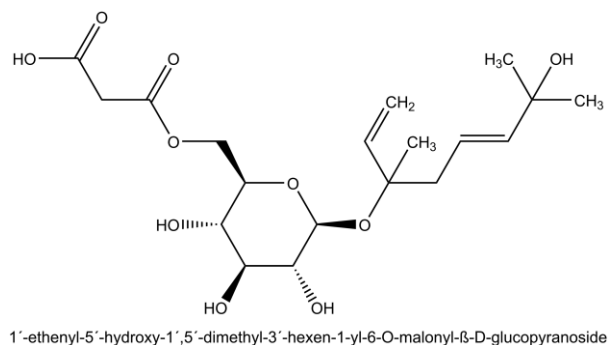
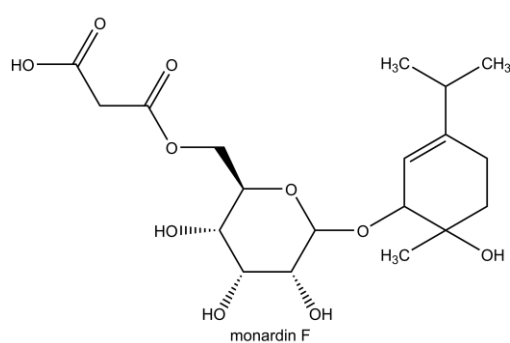
4D



4E



4F



4G

Fig. 3.4 Proposed chemical structures and fragmentation patterns of some compounds identified by our metabolic profiling to be associated with allelopathy (A, B, C, E) and compounds found in databases that correspond to putatively allelopathic compounds (D, F, G). ESI+/- (bolded) - fragment identified in both chromatographic systems (acetonitrile as well as methanol mobile phase systems), ESI+/- fragment identified only in one chromatographic system.

Supplementary material

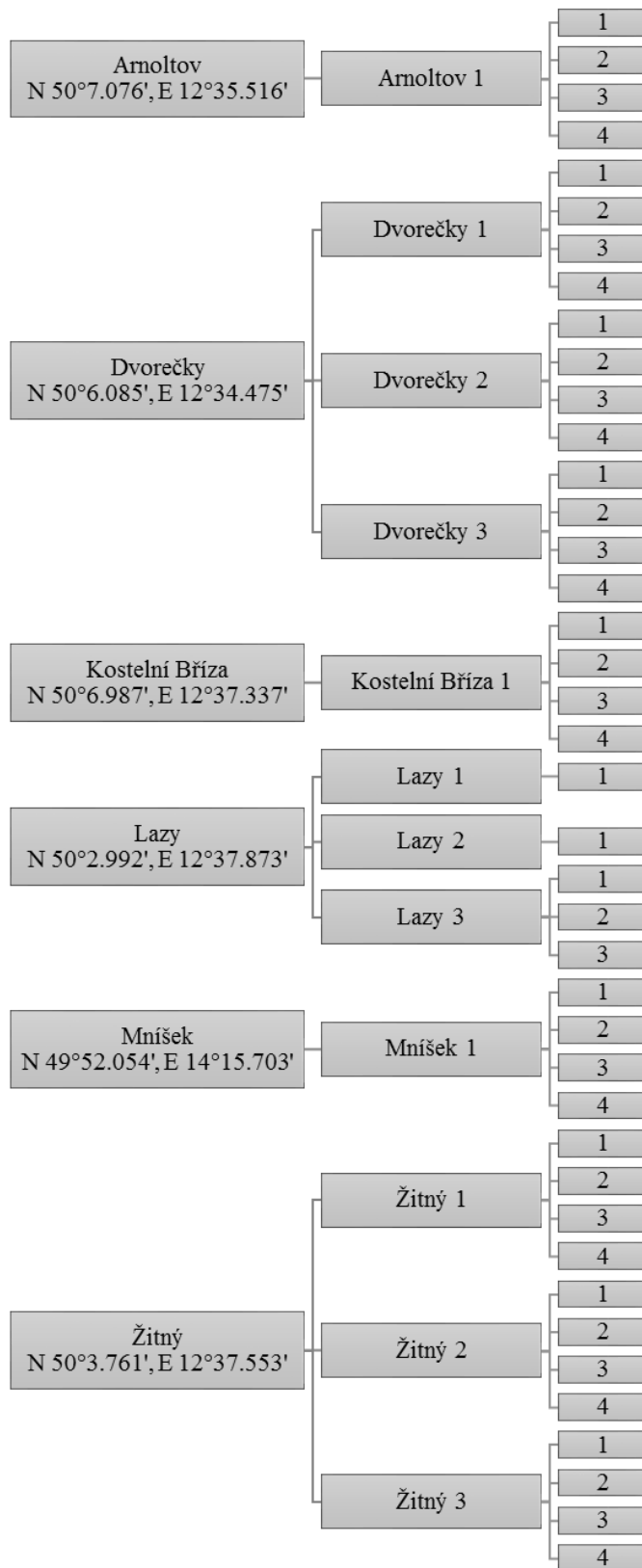


Fig. S3.1 Scheme of areas with GPS co-ordinates, populations within areas and maternal lines within populations sampled for seeds

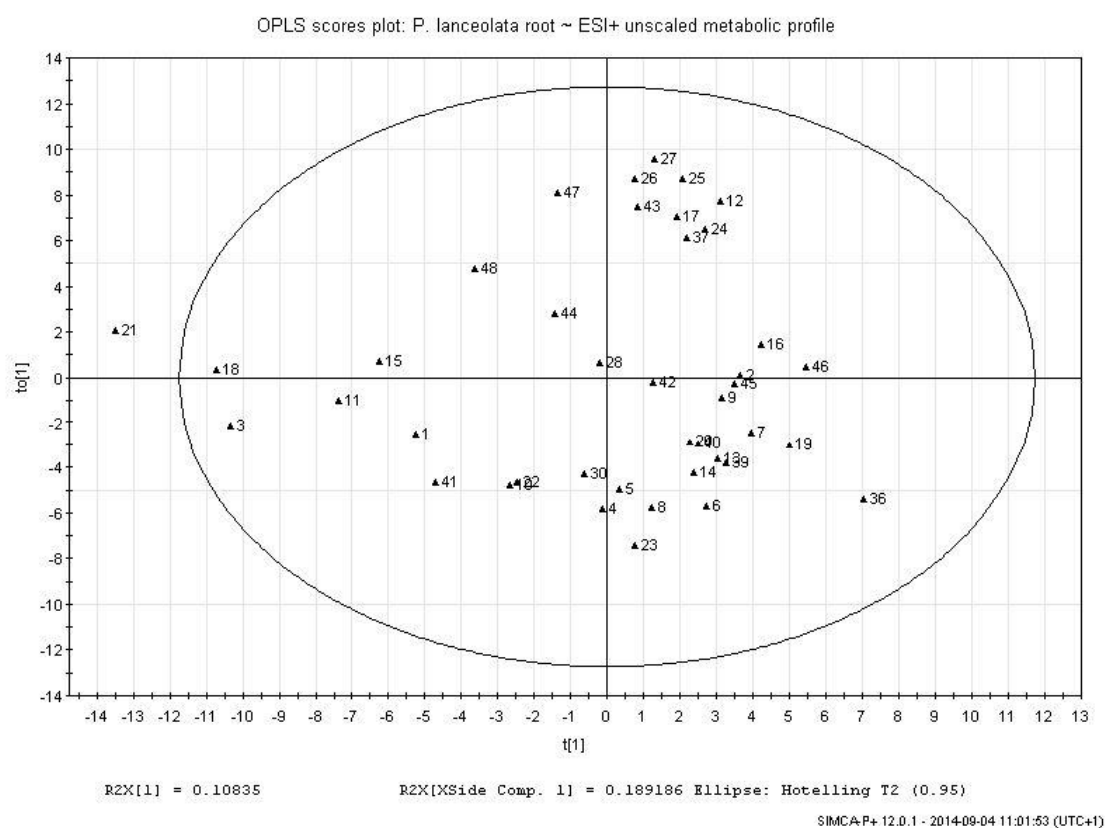


Fig. S3.2 Scores plot of an orthogonal partial least-square (OPLS) model explaining effect sizes of *P. lanceolata* root length in the bioassay by metabolomic data measured in positive ionisation mode (ESI+). The x-axis represents the predictive component of the OPLS model; the y-axis represents orthogonal systematic variation. The observation out of the ellipse was shown to have significantly higher influence (P -value < 0.01) on our models based on Hotelling's T2.

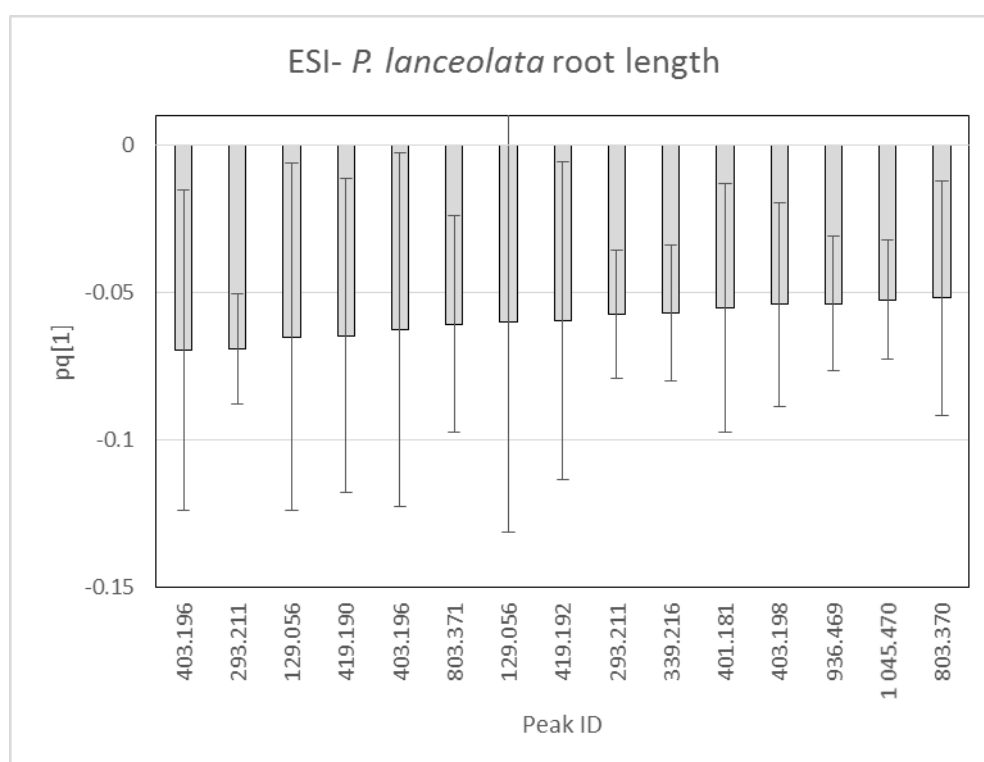


Fig. S3.3 Loading column plot of an orthogonal partial least-square (OPLS) model that remained significant after exclusion of the outlying maternal line. The plot shows metabolomic peaks (characterised by retention time $\times$ accurate m/z) most associated with the allelopathic effect that were extracted from the analyses of full metabolomic data. On the x-axis, the peaks are represented by its m/z values. The y-axis represents the predictive components of the OPLS model. Negative values of predictive components are associated with negative values of effect sizes in the bioassay, *i.e.* allelopathy. The error bars were calculated by jack-knifing (Efron and Gong 1983) in SIMCA-P software.

Table S3.1 Alternative analysis of proportions of total variance (%) in allelopathic effects of *H. mantegazzianum* attributed to variation among areas, among populations within areas, among individual maternal lines within populations and experimental variation. Only the areas Dvorečky and Žitný were analysed, since there was a perfectly balanced sampling design in these two areas with 3 populations within each area and 4 maternal lines within each population.

	Among areas	Among populations	Within populations	Residuals
Germination of <i>P. lanceolata</i>	<0.0001	<0.0001	15.6	84.4
Root length of <i>P. lanceolata</i>	<0.0001	<0.0001	47.0	53.0

Acknowledgements

This study was funded by grant P504/10/0132 from the Czech Science Foundation and grant GAUK512712 from the Charles University Grant Agency. Zdeněk Kameník was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project CZ.1.07/2.3.00/30.0003. Frederick Rooks kindly improved our English. We are grateful for the assistance of Tereza Klinerová, Dana Parysová, Daniel Samek, Alexandra Špaldoňová and Tereza Tylová. We thank Hans Albert Pedersen and two anonymous reviewers for insightful comments on the manuscript.

Long-term impact of *Heracleum mantegazzianum* invasion on soil chemical and biological characteristics

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SOIL BIOLOGY AND BIOCHEMISTRY (2014) 68: 270–278

Abstract

Exotic plant invasions often change ecosystem properties with subsequent impacts on the structure of invaded communities. Despite an increasing knowledge of post-invasion ecosystem changes, these modifications are only rarely studied within the temporal context of ongoing invasions. In this study we investigated 19 soil chemical and biological characteristics, as well as light conditions, in uninvaded grassland sites and compared them with those from sites invaded by giant hogweed (*Heracleum mantegazzianum*) for different times (from 11 to 48 years). We further related variation in these soil and light characteristics to richness and productivity of native plant species and hogweed cover measured in the field, and to common-garden performance of hogweed grown in soil inocula from the same sites.

Hogweed presence significantly reduced red/far-red light ratios but increased soil pH. Longer invasion history was associated with increasing soil conductivity and content of extractable phosphorus. There were also parameters that displayed opposite trends in different periods of invasion such as fungal/bacterial ratios or relative amount of photosynthetically active radiation (Δ PAR). These parameters initially increased (fungal/bacterial ratio) or decreased (Δ PAR), but after reaching a certain breakpoint they tended to return to pre-invasion conditions.

Differences in native species richness were best correlated with light availability and soil pH, and productivity with composition of soil microbial communities. Differences in hogweed cover were associated with soil pH and conductivity. The variation in hogweed

performance in a common garden was related to the composition of soil microbial communities, soil conductivity and light availability of sites from which soil inocula were collected.

This study documents that ecosystem properties can be altered not only by an invasion event but are further modified as the invasion proceeds. These ecosystem changes likely underlie long-term impacts of invasive plants on native communities.

Introduction

Impacts of invasive plants on ecosystem processes and properties are often coupled with those on the structure and composition of resident plant communities (Levine et al. 2003; Wolfe and Klironomos 2005; Hejda et al. 2009; Ehrenfeld 2010; Pyšek et al. 2012). Multiple mechanisms, including post-invasion changes in resource availability, composition of soil microbial communities or fire regimes, have been documented to reduce native species richness and modify the composition of plant communities (Levine et al. 2003). For example, species richness and abundance of native forest seedlings decreased in dense cover of *Tradescantia fluminensis*, an invasive weed in New Zealand podocarp forests, due to marked reduction of light availability (Standish et al. 2001). Two other exotic plants, *Alliaria petiolata* and *Chromolaena odorata*, were shown to suppress native plant growth by disrupting their mutualistic associations with arbuscular mycorrhizal fungi or by promoting the accumulation of soil fungal pathogens, respectively (Stinson et al. 2006; Mangla et al. 2007). Native shrubs in the western USA declined in communities invaded by exotic annual grasses due to an unprecedented increase of fire frequency (Mack 1989; D'Antonio and Vitousek 1992).

The above examples document that plant invasions can initiate ecosystem changes with significant effects on invaded communities. Invasion-altered communities may feed back (Ehrenfeld et al. 2005; Yelenik and Levine 2011; Seastedt and Pyšek 2011) to further modify ecosystem characteristics and thus the performance of native but also of exotic populations. These feedbacks then determine long-term dynamics within invaded communities (Bever et al. 1997; Cuddington and Hastings 2004). For example, positive feedbacks play a role in the success of the above-mentioned example of annual grass *Bromus tectorum* (D'Antonio and Vitousek 1992). Although it originally invaded interstices of shrubs (approximately a century ago), increased fire frequency promoted its dominance that in turn led to more fires, at the expense of native shrubland (reviewed by D'Antonio and Vitousek (1992). In contrast,

invasion-triggered ecosystem changes can promote the dominance of invaders over a short-term period, but further modification of biotic or abiotic conditions can be less favorable for the invaders than for the resident species, making co-existence of the exotics and natives possible over the long-term course (Yelenik and Levine 2011; Tang et al. 2012).

Surprisingly, although there is a large body of evidence revealing ecosystem and community impacts of invasive plants (e.g. Vilà et al. 2011; Simberloff 2011), for most of these systems information on the temporal scale of the changes is missing (discussed by Wolfe and Klironomos 2005 and Ehrenfeld 2010). There are few studies that document an invader's and native population dynamics, together with changes in ecosystem properties during different time points of invasion. For example, in the Great Lakes region, Mitchell et al. (2011) surveyed wetlands invaded by exotic cattails (*Typha* sp.) for different lengths of time. They found that cattail dominance (stem density) increased linearly from the beginning of the invasion, but there was a delay of 10 years before native diversity started to decline, likely due to litter accumulation.

It also remains underexplored whether the mechanisms promoting the dominance of invaders are identical with the mechanisms causing the native species to decline. Allison and Vitousek (2004) found faster decomposition and nutrient release from invasive than native litter in Hawaii. The authors hypothesize that novel soil nutrient conditions should favour exotics at the expense of natives that are less nutrient-limited. Levine et al. (2003) pointed out, however, that the decline of native species in Hawaiian ecosystem is not necessarily due to new dynamics of nitrogen cycling, but rather due to increased levels of shading in the presence of invaders. Usually many environmental factors change simultaneously during invasion and their relative contributions to community change should be distinguished; yet they are rarely examined in concert (Hulme et al. 2013).

In this study, we examined soil biological and chemical characteristics together with light availability at five uninvaded grassland sites that served as controls for 19 sites invaded by giant hogweed (*Heracleum mantegazzianum*) for different time periods ranging from 11 to 48 years in the Slavkovský les, the Czech Republic. In these same communities, native plant richness and native productivity was reduced by hogweed invasion and this negative impact peaked in grasslands invaded for ca. 30 years. At sites with a longer invasion history both parameters tended to recover whereas hogweed cover declined linearly over the whole period assessed. Interestingly, hogweed performance in a complementary common garden experiment declined in non-sterile inocula collected at sites with a longer invasion history (Dostál et al. 2013).

Here we analyzed the effect of hogweed invasion (i.e., presence/absence) and its invasion history (i.e., time since invasion) on 19 soil and light parameters: pH (water), pH (KCl), conductivity, extractable phosphorus, carbon, nitrogen, carbon/nitrogen ratio, relative amount of photosynthetically active radiation, red/far-red light ratio, arbuscular mycorrhizal neutral lipid fatty acids (NLFA), fungal phospholipid fatty acids (PLFA), bacterial PLFAs, actinobacterial PLFAs, G⁺ bacteria, G⁻ bacteria, total microbial biomass, fungal/bacterial ratio, G⁻/G⁺ ratio and ergosterol concentration. We also analyzed the effect of the invasion and of its history on the composition of soil microbial communities based on 19 PLFAs and NLFA detected at each site. We expected a post-invasion increase in available nutrients coupled with changes in soil microbial communities, but decrease in light availability.

In the second part of the study we used a subset of eight parameters, uncorrelated to each other, selected from the above characteristics, to investigate how they predict variation in native species richness and productivity, and hogweed performance that were studied by Dostál et al. (2013). We hypothesized that variation in native species richness and productivity would be related to changes in nutrient and light availabilities, whereas variation in hogweed performance would be related to differences in composition of soil microbial communities, indicating the presence of possible soil pathogen(s) of hogweed.

Methods

Study species

The giant hogweed (*Heracleum mantegazzianum*) is a monocarpic perennial umbellifer native to the Western Greater Caucasus (Russia, Georgia). It has spread in a number of European countries (Tiley et al. 1996; P Pyšek et al. 2007; Pyšek et al. 2008), Canada (Page et al. 2006) and the USA (Kartesz and Meacham 1999). It may form extensive, almost monospecific stands with negative effects on biodiversity (Pyšek and Pyšek 1995; Pergl et al. 2006; Thiele and Otte 2007; Hejda et al. 2009). The species is rich in secondary metabolites and especially known for linear furocoumarins that defend the plant against herbivory (Berenbaum 1981; Hattendorf et al. 2007) and for angular furocoumarins that serve as a defence mechanism against microbial infection (Fischer et al. 1978; Ivie 1978). Hogweed invasiveness is also attributed to allelopathic effects (Myras and Junttila 1981) but allelopathy is probably of minor importance for its dominance (Wille et al. 2013).

Study region

We performed our research in the Slavkovský les Protected Landscape Area, located in the western part of the Czech Republic, where the giant hogweed was introduced as a garden ornamental plant in the 19th century. The species started to spread after World War II, probably due to a lack of appropriate landscape management caused by displacement of German inhabitants and establishment of a military zone (Müllerová et al. 2005; P Pyšek et al. 2007).

Müllerová et al. (2005, 2013) reconstructed the hogweed invasion dynamics in this region using a series of aerial photographs and identified sites invaded (i) in 1962 or earlier, (ii) between 1963 and 1973, (iii) between 1974 and 1991, (iv) between 1992 and 2006, together with (v) uninvaded sites. Using this information we carried out field surveys in 2010 and included 24 sites from 5 areas differing in invasion history in this study (each community type was thus represented by 5(4) replicates; see Appendix 1). We estimated mean age of the hogweed stands at each site, yielding a chronosequence of invasive populations of a differing age, i.e. (i) a minimum of 48 years, (ii) 42 years, (iii) 28 years, (iv) 11 years and (v) 0 years (indicating hogweed-free sites).

Soil chemical characteristics

At each of 24 sites, we set up 3 transects (separated by 7 m) with three 2 x 2 meters plots per transect in June 2011. At several sites we were only able to install two transects due to the linear shape of the hogweed populations growing, e.g., along road edges. The sampling was thus done in 9 or 6 plots per site. These plots served to estimate native species richness and productivity, and hogweed cover (in %) at invaded sites (see Appendix 1). During the vegetation surveys soil samples of 100 cm³ were collected from 0-10 cm soil profiles in the same plots; they were further pooled to obtain one composite sample per site, air-dried, sieved on 2 mm mesh and subjected to following analyses.

The pH was measured according to ISO 10390:2005 in a 1:5 (volume to volume) suspension of soil in water and soil in 0.1M KCl using a glass electrode. The specific electrical conductivity was measured according to ISO 11265:1994 in 1:5 (weight to volume) aqueous extracts. Mehlich 3-extractable phosphorus was determined spectrophotometrically by the ammonium-molybdate ascorbic acid method at 750 nm (Genesys 10, Thermo Scientific, USA). Contents of total carbon and nitrogen were measured with a CHN elemental analyser (Carlo Erba NC2500, Italy). As the soil samples have been proved to be carbonate

free by the carbonate qualitative test (known as the “fizz” test), the total carbon stands for the organic carbon fraction (Peverill et al. 1999).

Soil microbial community

To assess the composition of soil microbial communities, soil samples of the same localities except “Lazy” area were analyzed (n = 20 sites; see Appendix 1). In August 2012, we set up two transects per site 14 m apart with three 2 x 2 m plots per transect separated by 10 m. In each plot we took a soil sample of 100 cm³ from 0-10 cm soil profiles. Samples were then pooled to obtain one composite sample per site and frozen immediately after sampling. When brought to the laboratory (1 – 2 days after sampling), the frozen samples were freeze-dried and sieved on 2 mm mesh prior to analyses. All analyses were performed in triplicate and mean values were used for statistical analyses.

Phospholipid (PLFA) and neutral lipid (NLFA) fatty acids were determined as biomarkers of composition of soil microbial communities. The soil was extracted by a mixture of chloroform–methanol–phosphate buffer (1:2:0.8) according to Bligh and Dyer (1959). Lipids were separated into neutral lipids, glycolipids and phospholipids using silicic-acid solid-phase extraction cartridges (LiChrolut Si 60, Merck). Neutral and phospholipid fractions were collected and transesterified with mild alkaline to obtain methyl esters of respective fatty acids and these were analyzed by gas chromatography–mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA, USA) following the method previously published in Šnajdr et al. (2008). Methylated fatty acids were identified according to their mass spectra and quantified using a mixture of authentic chemical standards obtained from Sigma. When referring to different fatty acids, rules described in Frostegård et al. (1993) were followed.

In spite of concerns regarding the specificity of categorization by particular fatty acids, discussed for example in Frostegård et al. (2011), here we have indicated different microbial groups following subsequent criteria. These PLFAs were summed up to estimate bacterial biomass: i14:0, i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7, 16:1 ω 5, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1 ω 7, 10Me-18:0 and cy19:0 (Frostegård and Bååth 1996; Bååth and Anderson 2003). PLFAs 10Me-16:0 and 10Me-18:0 were used to calculate the biomass of actinobacteria (Frostegård et al. 1993). 10Me-17:0 was excluded from further analyses as it occurred at three sites only. The PLFAs i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0 were used to calculate the biomass of G⁺ bacteria, and 16:1 ω 7, 16:1 ω 5, cy17:0, 18:1 ω 7 and cy19:0 were used to calculate the biomass of G⁻ bacteria (Šnajdr et al. 2008). The PLFA 18:2 ω 6,9 was

used as a marker for fungal biomass (Kaiser et al. 2010). We relied on NLFA 16:1 ω 5 to indicate arbuscular mycorrhiza rather than on PLFA 16:1 ω 5, which is a good indicator of AM in roots but not in soil (Olsson 1999; Hedlund 2002). Total content of all determined PLFA molecules was used as a measure of total microbial biomass. The fungal/bacterial ratio was calculated as fungal PLFA/bacterial PLFAs.

To support the estimation of fungal abundance, another membrane-bound substance specific to fungi, ergosterol, was extracted and determined as described in Šnajdr et al. (2008) using a Waters Alliance HPLC system (Waters, USA) with methanol as a mobile phase at a flow rate of 1 ml/min and UV detection at 282 nm. The ergosterol content was correlated to the PLFA 18:2 ω 6,9 ($R = 0.772$, $P < 0.001$).

Measurements of light conditions

In the same plots where we sampled soil for the analysis of soil microbial community ($n = 20$ sites) we also measured light conditions. In five randomly selected positions per plot, we measured red/far-red light ratio (R/FR ratio) at 5 cm above the ground and photosynthetically active radiation at 5 cm above the ground and 20 cm above the vegetation. In further analyses, we used measured R/FR ratio, and relative amount of photosynthetically active radiation (Δ PAR) obtained by dividing light quantity above the ground by light quantity above the vegetation. Smaller values of R/FR ratio and of Δ PAR indicate the presence and proximity of neighbours competing for light (Franklin 2008). We did light measurements using a SPh 2020 Photometer (Optické dílny ASCR, Turnov, the Czech Republic).

Statistical analyses

We performed two types of statistical analyses. First, we analyzed the effect of hogweed invasion (hogweed presence or absence) and of hogweed invasion history on soil chemical properties (pH (water), pH (KCl), conductivity, extractable phosphorus, carbon, nitrogen, carbon/nitrogen ratio), light conditions (Δ PAR, R/FR ratio) and the soil microbial community (arbuscular mycorrhizal NLFA, fungal PLFA, bacterial PLFAs, actinobacterial PLFAs, G+ bacteria, G- bacteria, total microbial biomass, fungal/bacterial ratio, G-/G+ ratio and ergosterol concentration). To explore invasion history, we used hogweed residence time, which was entered as linear or as linear and quadratic terms. Numerous studies indicate the existence of a threshold or breakpoint where natural systems respond rapidly to a relatively small change in a driver (Dodds et al. 2010; Elgersma and Ehrenfeld 2011). Therefore we also searched for a possible threshold effect due to hogweed presence or due to hogweed invasion

history. Based on visual inspection of the data, for pH (water), pH (KCl), extractable phosphorus, R/FR ratio, Δ PAR and fungal/bacterial ratio we ran an additional model with a threshold identified by an iterative search procedure (Crawley 2007).

For each environmental variable we ran 4 or 5 linear models, including models with intercept only. The model with the best fit was identified based on AICc, Akaike Information Criterion corrected for small sample size. Each site was represented by the mean value of respective soil chemical, biological or light characteristics. Hogweed management was initiated at six sites in 2011 leading to bias in light measurements. For these sites we therefore used values of Δ PAR and R/FR ratio obtained by regression of the two variables against estimated mean age of the hogweed stands. Prior to analyses, all environmental variables were inspected and, if necessary, square-root- or log-transformed to meet the assumptions of homoscedasticity and normality of residuals.

In addition, we performed principle component analysis (PCA) to see if the sites could be grouped based on the composition of their soil microbial communities. For that we used concentrations of all 19 phospholipid and neutral lipid fatty acids detected at each site. Further we conducted redundancy analysis (RDA) testing the effects of hogweed invasion and its history on composition of soil microbial communities. A Monte-Carlo permutation test (with 999 permutations within blocks defined by areas) was used to assess the significance of hogweed presence and of its invasion history. In both PCA and RDA analyses we used relative amounts of fatty acids within samples obtained by data centering and standardization by samples (*sensu* Lepš and Šmilauer 2003).

Second, we searched for which of the above-mentioned soil and light parameters explained the most variation in the native community and in hogweed performance. Specifically, in the univariate analyses, as responses we used native species richness and native productivity (site means, $n = 20$) obtained during vegetation surveys in 2011 as described above (Dostál et al. 2013). As a parameter of the hogweed performance we used its cover (in %) measured in the field. We also used hogweed biomass from a common garden experiment conducted during 2010-2011. In this experiment, hogweed was cultivated in soil inocula from the sites where the above-described soil and light parameters were measured. Brief description of this experiment can be found in Appendix 2 and more details are present in Dostál et al. (2013). We decided to use this additional measure of hogweed performance because hogweed eradication started at six sites in 2011 (see Appendix 1) and the information on hogweed cover from these localities could not be used in the analyses. The second reason for using the experimental results was that we could assess the effects of soil chemical and

biological characteristics on hogweed performance separately for plants cultivated in sterile (mean total biomass per inoculum origin, $n = 20$) and non-sterile inocula (mean total biomass per inoculum origin, $n = 18$). Significant effects of soil biological characteristics on hogweed performance were expected for the latter but not former treatment since Dostál et al. (2013) found hogweed biomass to decrease with the soil inoculum collected in the older hogweed stands but only if not treated by sterilization.

To avoid multi-collinearity in the analyses, only factors with correlations less than 0.7 were used as predictors (as suggested by Gujarati (1995), see correlation matrix in Appendix 3). This reduced the potential explanatory variables to 8 parameters: pH (KCl), conductivity, extractable phosphorus, nitrogen, carbon/nitrogen ratio, Δ PAR and composition of soil microbial communities expressed by the site scores of the first and the second axes of principal component analysis, PCA axis 1 and PCA axis 2. For each response variable (field native richness and productivity, field hogweed cover, experimental hogweed biomass) we thus started with all factors (full model) and eliminated non-significant ones by likelihood-ratio test to achieve the minimum adequate model. We used linear mixed-effects models with the above-listed factors as fixed terms and area ($n = 4$; Appendix 1) as a random term. Factors were standardized to zero mean and unit variance to facilitate comparisons of effect size estimates (Schiegg 2010).

We also performed multivariate redundancy analysis (RDA) of native plant and soil microbial composition. In RDA of native plant composition we used identical factors as in the above-described univariate analyses. In the case of RDA of soil microbial composition we used PCA axis 1 and PCA axis 2 site scores of vegetation, in addition to the soil chemical and light characteristics described above. The forward selection was used to test the significance of each factor by a Monte Carlo permutation test (with 999 permutations within blocks defined by areas). Only significant variables ($P < 0.05$) were included in the final RDA model. Data centering and standardization by samples (*sensu* Lepš and Šmilauer, 2003) was performed prior to the analyses.

All univariate analyses were performed in R version 2.14.2 (R Development Core Team 2012). Mixed-models were done with help of R package *lme4* (Bates et al. 2012). Multivariate analyses were performed using CANOCO 4.5 (Ter Braak and Šmilauer 2002).

Results

Effect of hogweed invasion and its invasion history on soil chemical and biological characteristics and light availability

Hogweed invasion or its history significantly, or marginally significantly, influenced seven of the 19 soil and light characteristics measured (Table 1). The soil pH (water, KCl) was higher at invaded (pH (KCl): 5.67 ± 0.10) than intact sites (5.07 ± 0.21 ; mean $\pm$ SE) whereas R/FR ratio decreased at invaded sites (0.47 ± 0.03 vs 0.69 ± 0.07 at uninvaded sites; mean $\pm$ SE). Δ PAR was also reduced by 56% on average in hogweed stands in comparison with uninvaded sites but it changed during invasion history: according to the model with linear time including a threshold, Δ PAR dropped at the beginning of invasion (i.e. at sites invaded for 11 years) but increased at sites invaded for a longer time (Table 1, Fig. 1).

The model with linear time was top-ranked in predicting change in conductivity and phosphorus as both parameters increased with longer residence time of hogweed (although its effect on phosphorus was only marginally significant; Table 1, Fig. 1). Fungal/bacterial ratio changed according to a quadratic model as it increased until hogweed residence time of 28 years, when it started to decline (Table 1, Fig. 1).

Regarding the composition of soil microbial communities, the first two principal components (PCA axis 1 and PCA axis 2) explained 94.4% of the variation (Fig. 2; Appendix 4). However, the hogweed presence or hogweed invasion history were not significant predictors of differences in soil microbial community as found by RDA (not shown).

Factors correlated with native plant community structure and with hogweed performance

Univariate analyses

Variation in native species richness measured in the field was influenced by two factors. Specifically, diversity declined with increasing pH (KCl) and increased with greater Δ PAR. Differences in native productivity were related to the differences in soil microbial communities as productivity decreased with higher PCA axis 2 scores of soil microbial composition. The fatty acids 10Me-16:0 (actinobacteria), 16:1 ω 7 and cy17:0 (both G-bacteria; Appendix 4) were found to be important due to their high PCA axis 2 scores.

Hogweed cover measured in the field was positively related to pH (KCl) but negatively to conductivity (Table 2; Fig. 3). Composition of soil microbial communities influenced hogweed performance in the common garden experiment. Specifically, the biomass of hogweed cultivated in non-sterile soil inocula was negatively correlated with PCA axis 1 scores of the communities. The high PCA axis 1 scores were found for 16:0, cy19:0 (G-

bacteria), i15:0 and i15:0 (both G⁺ bacteria; Appendix 4) fatty acids, indicating their importance. Hogweed biomass was also greater in soil inocula from sites with larger Δ PAR but decreased with higher conductivity (Table 2; Fig. 3). Biomass of hogweed grown in sterile soil inocula was not related to any factor included in the model.

Multivariate analyses

Redundancy analysis (RDA) of vegetation composition identified Δ PAR ($F = 2.89$, $P = 0.002$) and pH (KCl) ($F = 2.21$, $P = 0.024$) to be the significant factors, explaining 19.2% and 8.6%, of the variation in vegetation composition, respectively.

RDA of the composition of soil microbial communities identified the carbon/nitrogen ratio ($F = 2.83$, $P = 0.043$) to be the only significant factor and explained 15.9% of the variation in the data.

Discussion

In this study we demonstrated significant changes in several ecosystem characteristics following the invasion of an exotic plant species, *Heracleum mantegazzianum*. Importantly, there were significant differences not only between uninvaded and invaded sites, but in several parameters the sites also differed depending on how recently they were invaded. The results of the second part of this study further suggest that some of these ecosystem properties underlie the changes in native plant communities and hogweed performance.

As the study was based on an observational approach, we cannot distinguish whether the modification of ecosystem characteristics was due to the invasion of the exotic species with its novel traits (e.g. Wolfe and Klironomos 2005; Wardle et al. 2011), or rather due to the loss of native species displaced from invaded communities, a phenomenon previously documented for this study system (Pyšek and Pyšek 1995; Thiele and Otte 2007; Hejda et al. 2009; Dostál et al. 2013). We also cannot rule out the possibility that the invasion dynamics have been determined by the environmental differences already present before the invasion started. We consider this possibility less likely, however, as invaded and uninvaded sites were shown to be similar in elevation (m a.s.l.), yearly sum of solar radiation (kWh.m⁻²) and topographic wetness index (unitless) (Dostál et al. 2013).

There are several traits of *Heracleum mantegazzianum* that differentiate this species from native plants, and by being novel to the invaded communities they could contribute to the observed ecosystem changes. First, no native grassland species from the study system reaches comparable biomass: with stem height of 200–500 cm and leaves up to 250 cm in

length, it is the largest European forb (Tilley et al. 1996), restricting access to light for co-occurring plants. Second, hogweed can reach high dominance at invaded sites with cover up to 75% (Dostál et al. 2013). It is a monocarpic, deep-rooting perennial and non-clonal forb whereas intact grasslands are typically dominated by clonal perennial grasses (e.g. *Dactylis glomerata*, *Festuca* spp. and *Poa* spp.). Such contrasting life-history strategies can be associated with differences in litter decomposition rates or with variation in phenology and the vertical distribution of nutrient uptake, although direct measurements of these processes in native dominants and hogweed have not been undertaken so far. Finally, *H. mantegazzianum* produces a great diversity of secondary compounds, e.g. flavonoids, essential oils and furocoumarins (Tilley et al. 1996; Hattendorf et al. 2007) and some of these metabolites are likely to be novel to the invaded communities. These three distinct characteristics and possibly others not mentioned here (see Pyšek et al., 2007 for a summary of traits contributing to this species' invasion success) could have been responsible for the modification of the soil environment and light conditions in the invaded communities.

Effect of hogweed presence and its invasion history on soil chemical and biological characteristics and light availability

Though the majority of measured parameters, namely those describing soil biological characteristics, were not altered by hogweed invasion, there were three principal trends with respect to the post-invasion temporal changes. First, parameters such as R/FR ratio or pH decreased and increased, respectively, in response to hogweed invasion but did not further change during the ongoing invasion. Second, invasion induced a change and this divergence from pre-invasion conditions continued as the invasion proceeded. This scenario was observed for conductivity and phosphorus. Finally, some of the measured parameters (Δ PAR, fungal/bacterial ratio) were initially altered by the hogweed invasion but tended to return to pre-invasion conditions in older hogweed stands. These different trends demonstrate that considering differences between invaded and uninvaded plots without reference to invasion history can yield a misleading picture of the invasion's impact on ecosystem characteristics. Below we discuss in detail some ecosystem parameters in the context of hogweed invasion.

The decrease in light availability (i.e. Δ PAR) and light quality (i.e. R/FR ratio) was among the most profound changes detected at invaded sites. Our findings correspond with other studies documenting reduced light levels in the understory of invasive plants (e.g. Standish et al. 2001; Reinhart et al. 2006). Dense and extensive populations and large size resulted in only a small fraction of full light, in the most extreme conditions as little as 7.5%,

reaching the ground in hogweed stands. Δ PAR tended to increase in older hogweed stands, and that likely reflects decreasing hogweed cover found in the field (Dostál et al. 2013). It must be noted, however, that in the second part of this study we failed to prove the significant relationship between hogweed cover and Δ PAR, probably due to a small sample size ($n = 13$ invaded communities).

Given the large biomass of hogweed plants we also expected an increase in soil nitrogen and carbon, (Ehrenfeld 2003; Liao et al. 2008; Vilà et al. 2011). Nitrogen and carbon content did not, however, change following the invasion. The likely reason is that although hogweed productivity compensated for the post-invasion decrease in native biomass, it did not increase the overall productivity at invaded sites (Dostál et al. 2013). It must be noted, however, that we measured the content of soil nitrogen and carbon only once during the vegetation period. Although invaded and uninvaded sites were similar in soil nitrogen and carbon, there could still be differences in the nutrient supply and uptake dynamics linked to, for example, differences in the phenology of dominant natives and that of invasive species (Myras and Junttila 1981; Dickens et al. 2013). Nevertheless, Dassonville et al. (2008), who analysed soil nutrients altered by invasion of several species including *H. mantegazzianum*, also failed to find a consistent increase in soil carbon, nitrogen and other nutrients. They rather showed a site-specific impact, with nutrients increasing and decreasing at nutrient-poor and -rich sites, respectively.

The increase in phosphorus concentration at sites with a longer invasion history can be explained by nutrient uplift from deep soil layers by a deep-rooting species such as *H. mantegazzianum*, as reported by Dassonville et al. (2008). These authors also proposed that increased phosphorus can result from active mobilization by rhizospheric processes like those documented by Chapuis-Lardy et al. (2005), who found enhanced mineralization of phosphorus due to increased soil microbial activity at sites invaded by the exotic forb *Solidago gigantea*.

Similarly, conductivity also increased at sites invaded for a longer time. In a study of a related species, *Heracleum laciniatum*, elevated conductivity of root leachates was associated with phytotoxic effects (Myras and Junttila 1981). Although here we found greater conductivity in older hogweed stands, in a previous study from the same study sites Dostál et al. (2013) did not find the phytotoxic effects to be stronger in soil from invaded than uninvaded sites, or to increase with the residence time of giant hogweed in a site. Therefore, the link between conductivity and phytotoxicity remains unresolved.

In contrast to several previous studies (e.g. Kourtev et al. 2002; Lankau 2011; Elgersma and Ehrenfeld 2011) we did not find hogweed invasion or its invasion history to significantly modify the composition of soil microbial communities. The exception was fungal/bacterial ratio with a unimodal pattern during invasion history. Fungal/bacterial ratio is an important soil biological metric linked to carbon and nitrogen cycling, carbon storage and decomposition of soil organic matter (Six et al. 2006; Högberg et al. 2007; Cotrufo et al. 2009). In our study, differences in this ratio likely reflected changes in native communities and hogweed cover during its invasion history, with a linear decline of hogweed cover but unimodal change in native community richness and native productivity (Dostál et al. 2013). More experiments are needed to test, for example, how the fungal/bacterial ratio changes with varying proportions of hogweed and native species litter (e.g. Elgersma et al. 2011).

Factors correlated with native plant community structure and with hogweed performance

Whereas in the first part of this study we investigated whether and how invasion and its history changed ecosystem characteristics, in the second part we linked variation in these parameters with native community structure and hogweed performance, measured in the field and common garden experiment. We found that native species richness was positively related to aboveground resource availability, specifically to the availability of light (Δ PAR). This resource became much scarcer at the beginning of invasion but tended to increase as invasion proceeded. The increase in light availability probably explains the return of some grassland species that were initially displaced from invaded communities (Dostál et al. 2013). This explanation is supported by the significant effect of Δ PAR on vegetation composition. Soil pH, also shown to be altered by invasion, had the opposite effect on native species richness. Specifically, pH was positively correlated with hogweed cover but negatively with native species richness, and varied with native species composition. These effects on native community characteristics are hard to explain based on our data and require additional study.

Regarding hogweed performance in a common garden experiment, we showed that its biomass was negatively correlated with conductivity and positively with Δ PAR, i.e. light regimes at sites of soil inoculum origin. More importantly, the hogweed biomass was significantly related to the differences in soil microbial communities. In a previous study, Dostál et al. (2013) found decreasing hogweed biomass in non-sterile soil collected from sites with a longer invasion history but the decrease was not observed in sterile soil. We thus predicted that hogweed performance will change with the composition of soil microbiota, differentiated by the age of hogweed stands. Although the differences in the soil microbial

communities were not driven by the invasion history, they significantly predicted the variation in hogweed biomass. The composition of soil microbiota was significantly related also to the productivity of native species, indicating the importance of soil biological characteristics for the variation in both the native community and invader performance. Our results, however, suggest that different microbial groups are involved in these relationships.

Findings of our study suggest that hogweed removal from the communities, although potentially complicated by a persistent soil seed bank (Moravcova et al. 2006) and long-distance seed dispersal (Pergl et al. 2011), can immediately improve light conditions and thus favour the recovery of native species' richness. However, as we show here, native richness and productivity are also controlled by soil chemical and biological characteristics. In contrast to light availability, improvement of soil conditions following hogweed removal may be delayed, which is likely to prolong the full recovery of native communities (e.g. Zavaleta et al. 2001; Elgersma et al. 2011).

Table 4.1 ΔAICc scores for candidate models testing the effect of hogweed presence (Hogweed) or of its residence time (Time) on soil chemical and biological characteristics and light availability. Time was entered as a linear term only or as linear and quadratic terms. Six environmental parameters were also examined by the model with linear time including a threshold. Soil chemical characteristics were measured at 24 study sites and soil microbial composition and light conditions at 20 sites differing in hogweed presence and its invasion history (age of hogweed populations ranging from 11 to 48 years). Asterisks (or NS) indicate the best model (i.e., model with $\Delta\text{AICc} = 0$) and the significance of that model is provided (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; (*), $0.05 < P < 0.10$; NS, non-significant).

	Intercept	Hogweed	Time	Time, Time ²	Time + threshold	Adjusted R^2 for the best model
<i>Soil chemical characteristics</i>						
pH (H ₂ O)	1.316	(*)	1.961	1.719	2.353	0.113
pH (KCl)	4.159	*	2.159	1.957	2.918	0.212
Conductivity	11.498	9.268	***	2.905	-	0.419
Phosphorus	1.416	3.685	(*)	2.176	4.238	0.117
Carbon	NS	2.592	2.352	5.068	-	NS
Nitrogen	NS	2.599	2.244	4.724	-	NS
Carbon/nitrogen ratio	NS	2.628	2.599	5.395	-	NS
<i>Light conditions</i>						
Δ Photosynthetically active radiation	11.659	2.693	13.954	5.057	***	0.589
Red to far-red ratio	7.515	**	7.132	4.265	5.725	0.370
<i>Soil biological characteristics</i>						
NLFA16:1w5 (AM fungi)	NS	2.175	2.794	5.463	-	NS
Fungi	NS	2.790	2.673	5.656	-	NS
Bacteria	NS	2.188	2.020	5.130	-	NS
Actinobacteria	NS	0.412	2.169	4.933	-	NS
G+ bacteria	NS	1.785	2.097	5.129	-	NS
G- bacteria	NS	2.126	2.063	4.931	-	NS
Total PLFAs	NS	2.183	2.117	5.280	-	NS
F/B ratio	2.130	1.514	2.069	*	3.725	0.254
G-/G+ ratio	NS	2.793	2.670	4.767	-	NS
Ergosterol	NS	2.512	2.367	4.484	-	NS

Table 4.2 An overview of minimum adequate models showing significant factors explaining variation in native species richness and productivity and in hogweed biomass. Native species richness and productivity and hogweed cover were measured at 20 study sites of 4 areas; hogweed biomass comes from a common garden experiment that used soil inocula collected at the same study sites (see Methods for more details). Factors were selected out of 8 parameters: pH (KCl), conductivity, phosphorus, nitrogen, carbon/nitrogen ratio, Δ photosynthetically active radiation, PCA axis 1 and PCA axis 2 of fatty acids compositions detected by PLFA/NLFA analysis.

	Coefficient estimate	SE	t-value	P
<i>Field measurements</i>				
Native species richness				
Intercept	33.835	1.507	22.449	<0.001
pH (KCl)	-4.462	1.369	-3.260	0.006
Δ Photosynthetically active radiation	2.898	1.336	2.168	0.048
Native productivity				
Intercept	11.965	1.533	7.806	<0.001
PCA 2 (PLFA)	-2.922	1.232	-2.372	0.032
Hogweed cover				
Intercept	58.771	3.527	16.665	<0.001
pH (KCl)	8.177	1.975	4.141	0.006
Conductivity	-5.126	2.130	-2.406	0.052
<i>Common garden experiment</i>				
Hogweed biomass (non-sterile inocula)				
Intercept	3.296	0.301	10.921	<0.001
Conductivity	-0.399	0.134	-2.984	0.012
Δ Photosynthetically active radiation	0.362	0.154	2.348	0.039
PCA 1 (PLFA)	-0.410	0.167	-2.457	0.032
Hogweed biomass (sterile inocula)				
Intercept	5.057	0.201	25.142	<0.001

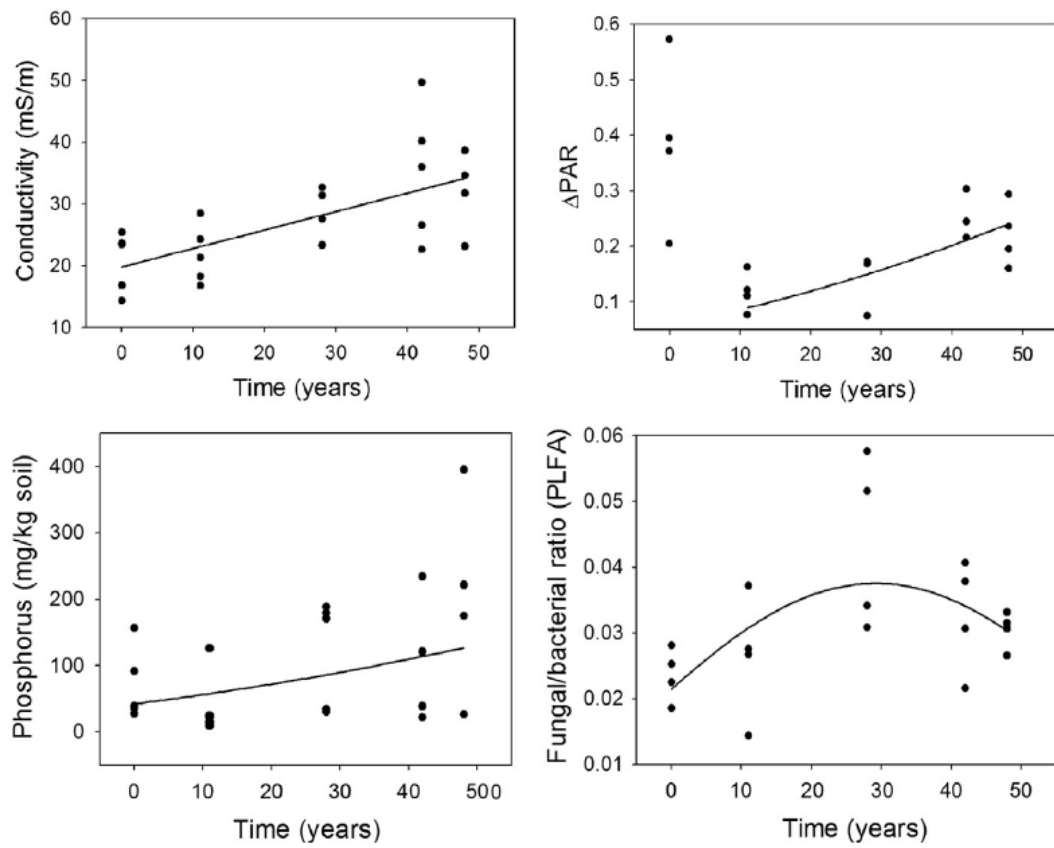


Fig. 4.1 Effect of hogweed invasion history on four selected characteristics measured at uninverted (hogweed residence time = 0 years) and inverted sites (residence time from 11 to 48 years). Points are site means and fitted lines come from the top candidate model with the lowest AIC (see Table 1). Δ PAR, relative amount of photosynthetically active radiation measured as ratio of light available at 5 cm above-ground and light available above vegetation.

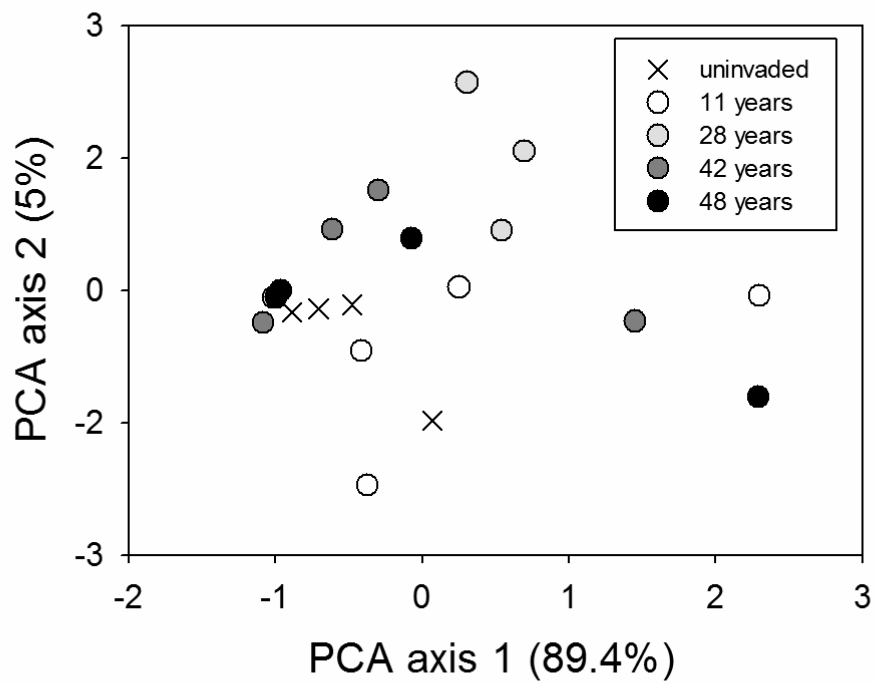


Fig. 4.2 PCA results for PLFA analysis of soil microbial communities of 20 sites differing in hogweed invasion history. Uninvaded sites as well as sites invaded for 11 to 48 years were sampled. PCA Axis 1 described 89.4% and PCA Axis 2 5.0% of variation in the data. Invasion history was not a significant predictor of the composition of soil microbiota (based on redundancy analysis).

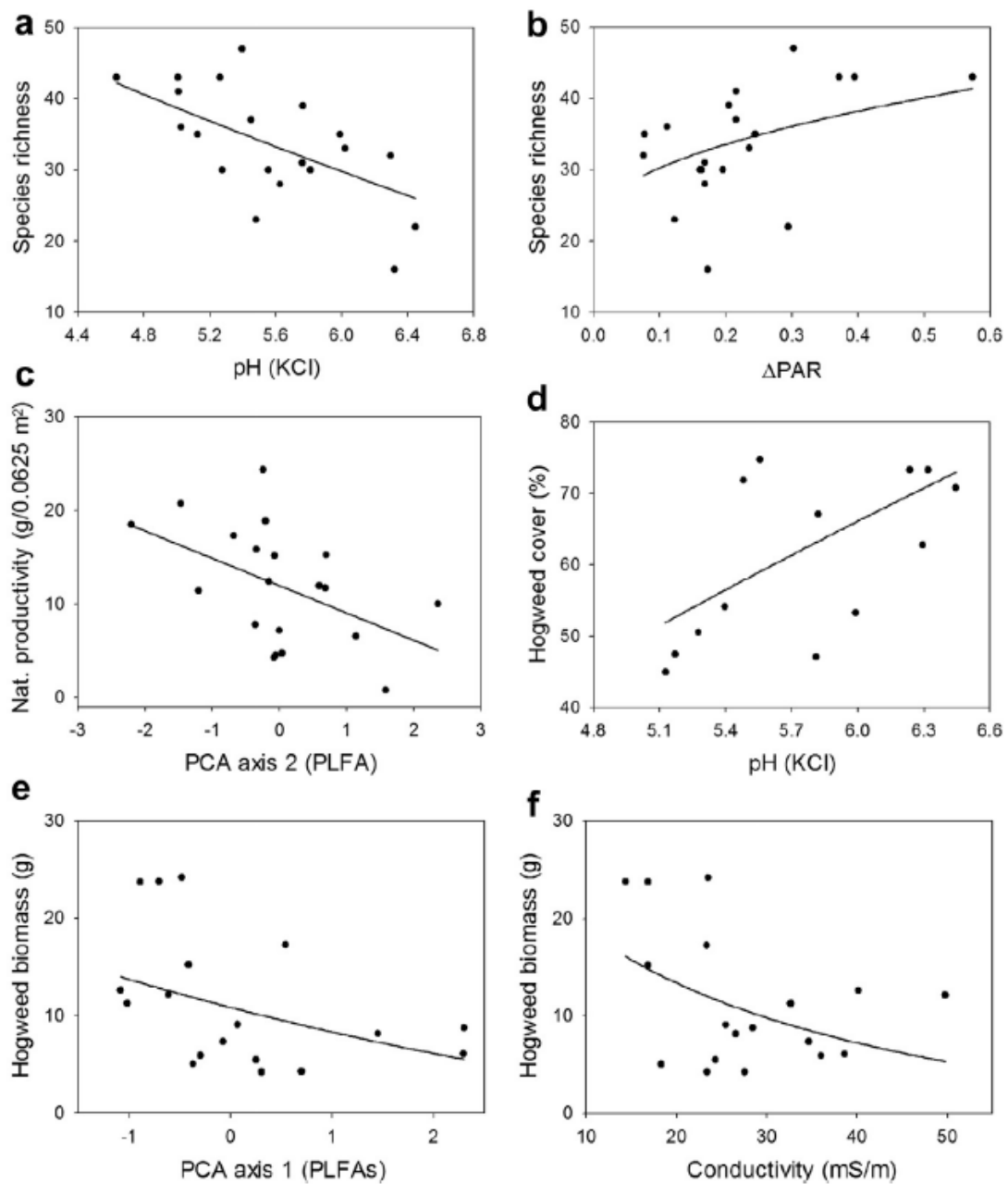


Fig. 4.3 Relationship of native species richness (a,b), native productivity (c), hogweed cover (d) measured in the field and hogweed performance from a common garden (e, f), and the environmental factors. Lines are fits from the minimum adequate models (all predictors significant at $P \leq 0.05$; for more details see Table 2). In the common garden experiment, hogweed was grown in non-sterile soil inocula that were sampled at sites where soil chemical and biological characteristics and light conditions were measured. Δ PAR, relative amount of photosynthetically active radiation measured as ratio of light available at 5 cm above-ground and light available above vegetation. PCA Axis 1 and Axis 2 (PLFA), site scores of soil microbial communities determined by PLFA.

Acknowledgements

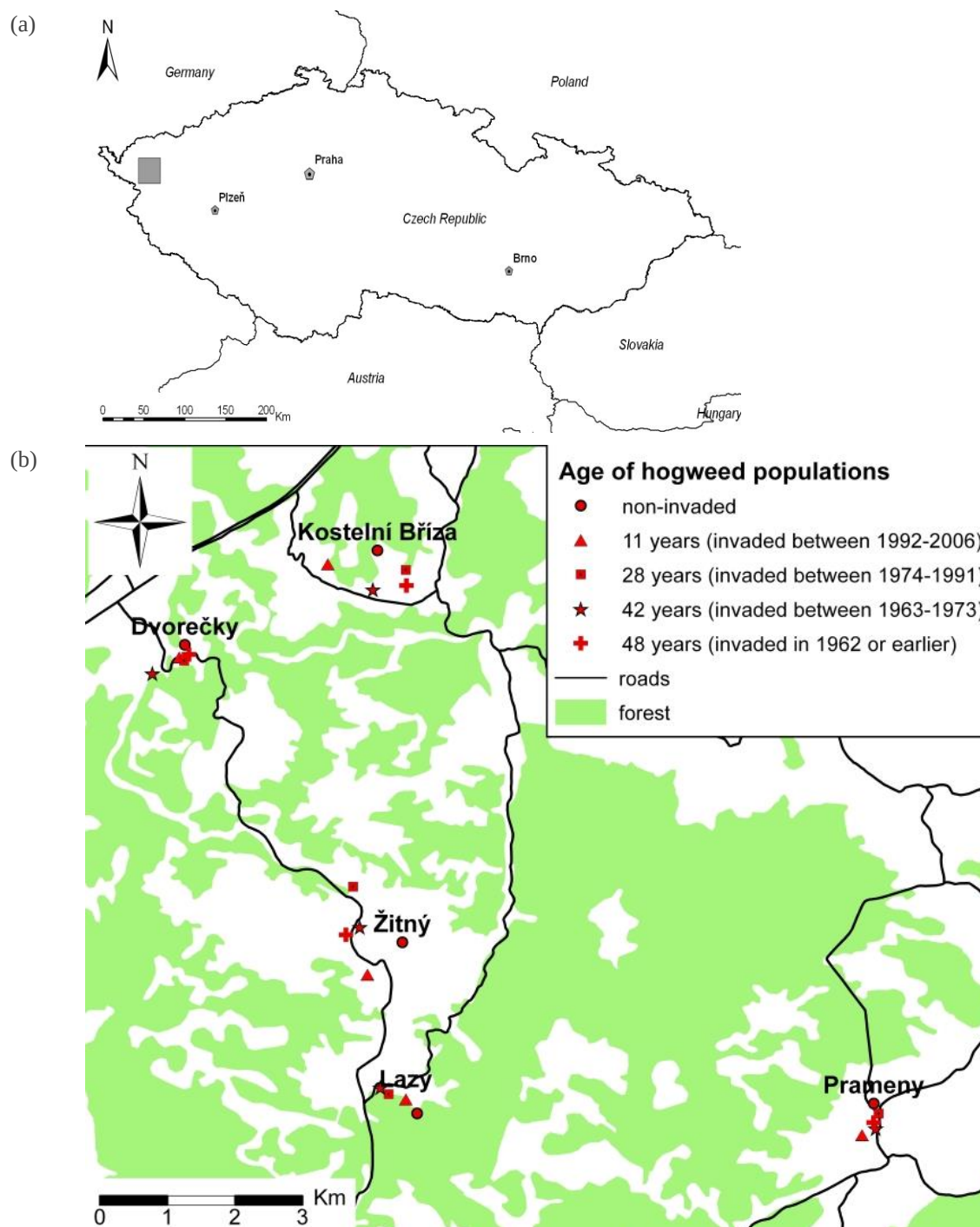
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Appendices

Appendix 4.1 An overview of the localities included in the survey of plant communities and soil chemical characteristics in 2011. All but the sites from the “Lazy” area were included in soil inoculum sampling for the common garden study initiated in 2010. These sites were also studied for composition of soil microbial communities and light conditions in 2012. Hogweed management was initiated at 6 sites in 2011 and light measurements from these sites would be biased. Instead, we obtained Δ PAR and R/FR ratio by regression against estimated mean age of the hogweed stands. Areas are named after settlements with geographical coordinates provided.

Area	Age of hogweed stands	Hogweed cover (%)	Native species richness (# of species)	Productivity (native biomass, g/0.0625 m ²)
Dvorečky (N50°06.104', E12°34.474')	0	-	43	18.87
	11	74.78	30	4.74
	28	73.33	16	0.77
	42	54.17	47	6.56
	48	70.83	22	7.17
Kostelní Bříza (N50°06.962', E12°37.289')	0	-	43	12.41
	11	managed	36	4.48
	28	managed	28	11.70
	42	53.33	35	15.26
	48	50.56	30	11.42
Lazy (N50°02.978', E12°37.867')	0	-	36	14.46
	11	73.33	30	12.97
	28	67.13	31	9.36
	42	47.50	28	19.19
Prameny (N50°02.992', E12°43.974')	0	-	43	20.74
	11	71.89	23	17.31
	28	managed	31	4.25
	42	managed	37	7.78
	48	managed	33	15.18
Žitný (N50°03.636', E12°37.582')	0	-	39	24.38
	11	45.00	35	18.49
	28	62.78	32	9.99
	42	managed	41	15.84
	48	47.11	30	11.96

(a) Map of the Czech Republic with a square indicating the study location. (b) Map of the sites included in the study. Within each area, there were uninvaded grasslands and grasslands invaded for 11 to 48 years.



Appendix 4.2 Brief description of a common garden experiment.

In this experiment we investigated the effect of soil sampled at sites with different invasion histories on hogweed performance. Soil samples were obtained at 20 sites (see Appendix 1; soil sampling was not performed in the “Lazy” area) and half of the soil from each site was sterilized by 25 kGy gamma irradiation, and the other half was left untreated. Sterile and non-sterile inocula were then used for hogweed cultivation during June 2010 till July 2011. Along with soil sterilization, the experimental treatments also included the addition of activated carbon and competition with native species, but these additional treatments were not considered in a present study. Here, we used the mean hogweed biomass per site of inoculum origin, separately for sterile and non-sterile soil inocula harvested in July 2011. Initially (June 2010), there were 360 pots with hogweed in total, one hogweed plant per pot. For a full description of the experiment, see Dostál et al. (2013): The impact of an invasive plant changes over time. *Ecology Letters* 16, 1277–1284.

Appendix 4.3 Correlation matrix of the explanatory variables. PCA axis 1 and PCA axis 2 stand for composition of microbial communities found by PLFA/NLFA analysis. Δ PAR, relative amount of photosynthetically active radiation measured as ratio of light available at 5 cm above-ground and light available above vegetation.

	pH (KCl)	Conductivity	Nitrogen	Phosphorus	C/N ratio	Δ PAR	PCA axis 1
Conductivity	0.294						
Nitrogen	0.043	-0.113					
Phosphorus	0.644	0.214	0.224				
C/N ratio	0.286	-0.057	0.007	0.477			
Δ PAR	-0.286	0.063	0.251	0.081	0.145		
PCA axis 1	-0.271	0.119	-0.067	-0.449	-0.534	-0.293	
PCA axis 2	0.565	0.217	0.026	0.211	0.294	-0.118	0

Appendix 4.4 PCA axis 1 and PCA axis 2 scores of 19 phospholipid and neutral lipid fatty acids detected at 20 study sites.

Organisms	Diagnostic fatty acids	PCA1 score	PCA2 score
Gram negative bacteria	16:1 ω 7	-0.8688	0.2227
	16:1 ω 5	-0.9028	0.1579
	18:1 ω 7	-0.0755	-0.2478
	cy17:0	0.234	0.2044
	cy19:0	0.7969	-0.5672
Gram positive bacteria	i14:0	-0.786	0.0382
	i15:0	0.6708	0.0891
	a15:0	0.6642	0.0115
	i16:0	-0.5598	-0.3599
	i17:0	-0.6092	-0.0267
	a17:0	-0.6462	-0.0657
Bacteria	17:0	-0.7403	0.1435
	15:0	-0.7778	0.0192
	16:1 ω 9	-0.7582	0.0594
Actinobacteria	10Me-16:0	0.5343	0.2374
	10Me-18:0	-0.2061	-0.1765
Fungi	18:2 ω 6,9	-0.7289	0.179
VAM fungi	16:1 ω 5 (NLFA)	-0.9799	0.0728
Others	16:0	0.978	0.1757

Summary and conclusions

The question why some introduced species become invasive in their new ranges is among the main concerns of today's ecology. It is directly related to the general question of species distribution, which has challenged academic minds for even longer. These questions can be approached from the population-level perspective, which describes a species' distribution as a result of competition between plants. In most cases, competition for resources such as nutrients, light and water is considered; however, non-resource-based competition has gained growing attention since its introduction into ecology in the 1960s. The so-called novel weapons hypothesis states that, in some invasive plants, the success of the species can be ascribed to the production of allelochemicals that increase its competitive ability. Secondly, there is the ecosystem-level perspective, which ascribes species' distributions to interactions with biotic and abiotic environmental factors. Some invasive plants became successful in their new ranges because they escaped its specialist herbivores, and as a result, they were able to allocate more resources into their competitive ability. Some also succeeded in their new ranges because they disrupted mutual associations of native plants with soil microorganisms to the detriment of native species and to the competitive advantage of the invaders. Changes in nutrient cycling caused by invasive plants were also observed to put native species at a disadvantage.

Apart from clarifying mechanisms acting in nature and thus gaining knowledge about the world, a better understanding of invasions is instrumental for developing efficient management strategies. *Heracleum mantegazzianum*, or giant hogweed, is a highly invasive species with a considerably negative impact on the biodiversity of entire ecosystems. Moreover, the species can be harmful to people as well as livestock, because it produces furanocoumarins causing photodermatitis. Therefore, effective management of the species is needed, but this requires deeper knowledge about the mechanism of its invasion success.

In this thesis, I provide a comprehensive and repeated test of the novel weapons hypothesis on the example of the *H. mantegazzianum* invasion. The invader was found to exert phytotoxic effects *in vitro* as well as in the garden experiment; however, these effects did not differ from the inhibition caused by the native species tested (Chapter 2). My results indicated that the allelopathic effect of *H. mantegazzianum* exudates in the species'

introduced range is not a constant trait, but a highly variable one. Whereas competitive ability or defence mechanisms are known to be variable at the genetic and environmental level, there is a lack of studies that document such variability in the field of chemically mediated plant-plant interactions. I therefore examined the intraspecific variability in allelopathy of *H. mantegazzianum* (Chapter 3). Variation partitioning among areas, populations within areas and maternal lines within populations revealed that the highest variance in allelopathic effects lies among maternal lines. Allelopathic effects in the bioassay were predicted by metabolic profiles of different maternal lines, and compounds associated with allelopathy were tentatively identified. Specifically, three groups of compounds were associated with allelopathy: (1) two dipeptides and one amino acid, (2) six C18 oxylipins, and (3) five malonyl-monoglycosides (Chapter 3). The composition of soil microbial communities did not change either as a consequence of the invasion event or during the invasion procedure. On the other hand, the invasion event significantly reduced red/far-red light ratios and increased soil pH. The observed ecosystem changes at sites invaded by *H. mantegazzianum* predicted variation in native species richness and productivity as well as the performance of giant hogweed itself (Chapter 4).

Although *H. mantegazzianum* has strong defences against herbivory and microbial infection, especially in its characteristic secondary metabolites (furanocoumarins), it does not seem to displace competing plants based on chemically mediated interactions. Accordingly, the compounds revealed to be associated with allelopathy were different from compounds previously reported to maintain the defence mechanism against herbivores and pathogens. I therefore suggest that different metabolic pathways are responsible for chemical defence and allelopathy, respectively. Future research will hopefully shed light on mutual relationships associated with particular chemically mediated interactions of plants. Moreover, I observed high intraspecific variability in several traits of the invader, and these may together create a highly plastic profile of the species that can contribute to its vast invasiveness. Trade-offs related to the production of allelochemicals in *H. mantegazzianum* need to be estimated in order to explain why the high within-population variability observed in our system is sustained.

To summarise, although root exudates of the invader *H. mantegazzianum* are able to inhibit native plant competitors, I found these effects to be comparable with the same effects of native species. Moreover, the composition of soil microbial communities was not substantially altered at sites invaded by the species. I therefore suggest that allelopathy through the production of unique and novel compounds, as predicted by the novel weapons

hypothesis, is not very likely to be a principal driver of the invasion success of *H. mantegazzianum*. I suggest that decreased light levels observed in stands infested by the species, together with mechanisms identified earlier, are responsible for the rapid spread and strong impact of the species on invaded communities. The results of this thesis therefore suggest that removal of giant hogweed would be an efficient strategy of management because no substantial invasion-related changes in the composition soil microbes were found in the field.

Overall, this thesis presents an interdisciplinary shot at testing the novel weapons hypothesis by using several different experimental methods and instrumental techniques. In particular, it brings strong evidence that untargeted metabolomics is an efficient tool to study chemically mediated plant-plant interactions whenever unknown metabolites are involved.

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PUBLICATIONS

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GRANTS

- 2014 Travel grant of Nadání Josefa, Marie a Zdeňky Hlávkových Foundation to attend the 7th World Congress on Allelopathy, Vigo, Spain
- 2013 Parkinson Travel Award to attend the 14th Soil Ecology Society Conference, Rutgers, Camden, USA
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- 7th WORLD CONGRESS ON ALLELOPATHY, 28 July – 1 August, Vigo, Spain. Oral presentation Intraspecific Variability in Allelopathy of *Heracleum mantegazzianum* is linked to metabolic profile
- 14th SOIL ECOLOGY SOCIETY CONFERENCE, 11-15 June 2013, Rutgers, Camden, USA. Oral presentation Long-Term Impact of Giant Hogweed Invasion on Soil Chemical and Biological Characteristics
- ICE-12: THE 12th INTERNATIONAL COURSE IN CHEMICAL ECOLOGY, 26 November – 7 December 2012, Max Planck Institute for Chemical Ecology, Jena, Germany. Poster presentation Allelopathy and Giant Hogweed Invasion
- NEOBIOTA 2012: Halting Biological Invasions in Europe: from Data to Decisions 7th European Conference on Biological Invasions Pontevedra (Spain), 12-14 September 2012; Poster presentation The Role Of Allelopathy in *Heracleum mantegazzianum* Invasion

MEMBERSHIPS

Soil Ecology Society (SES); International Allelopathy Society (IAS)