

1 **Novel chemistry of invasive plants: exotic species have more unique**
2 **metabolomic profiles than native congeners**

3

4 Mirka Macel^{1,2,3*}, Ric C.H. de Vos^{4,5}, Jeroen J. Jansen^{1,6}, Wim H. van der Putten^{1,7}, Nicole M.
5 van Dam^{2§}

6

7 ¹ Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box
8 50, 6700 AB Wageningen, The Netherlands

9 ² Molecular Interaction Ecology, Institute of Water and Wetland Research (IWWR), Radboud
10 University Nijmegen, P.O. Box 9010, 6500 GL, Nijmegen, The Netherlands

11 ³ Plant Ecology, University of Tübingen, Auf der Morgenstelle 3, 72076 Tübingen, Germany.

12 *Correspondence: Tel: +49-7071-2978814, Fax:+49-7071-295356, email: mirka.macel@uni-
13 tuebingen.de

14 ⁴ Plant Research International, Wageningen University and Research Centre (WUR), P.O. Box
15 17, 6700 AA Wageningen, The Netherlands. ric.devos@wur.nl

16 ⁵ Centre for BioSystems and Genomics, P.O. box 98, 6700 AB, Wageningen, The Netherlands

17 ⁶ Department of Analytical Chemistry, Radboud University Nijmegen, Heyendaalseweg 135,
18 6525 AJ Nijmegen, The Netherlands. jj.jansen@science.ru.nl

19 ⁷ Laboratory of Nematology, Wageningen University and Research Centre (WUR), P.O. Box
20 8123, 6700 ES Wageningen, The Netherlands. w.vanderputten@nioo.knaw.nl

21 [§]New Address: German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig,
22 Deutscher Platz 5e, D-04103 Leipzig, Germany. nicole.van_dam@idiv.de

23 **Authorship** MM designed, performed and analyzed the research and wrote the manuscript.
24 WHvdP and NMvD advised on the design and contributed to the writing. RCHdV performed the
25 metabolomics analyses and helped with data interpretation, JJJ contributed to the data analysis.

26
27 **Running title:** Novel metabolomic profiles of exotic plants

28
29 **Key words:** metabolomics, novel weapons, LC-MS, secondary metabolites, range expanding,
30 herbivory, *Solidago*, *Senecio*, *Asteraceae*, *Mamestra brassicae*

31
32 **Type of article:** Letter

33
34 **Words abstract:** 150

35
36 **Words main text:** 3901

37
38 **Number of references:** 50

39
40 **Figures:** 3, **Tables:** 3

41

42

43

44

45

46 **Abstract**

47

48 It is often assumed that exotic plants can become invasive when they possess novel secondary
49 chemistry compared to native plants in the introduced range. Using untargeted metabolomic
50 fingerprinting, we compared a broad range of metabolites of six successful exotic plant species
51 and their native congeners of the family Asteraceae. Our results showed that plant chemistry is
52 highly species-specific and diverse among both exotic and native species. Nonetheless, the exotic
53 species had on average a higher total number of metabolites and more species-unique
54 metabolites compared to their native congeners. Herbivory led to an overall increase of
55 metabolites in all plant species. Generalist herbivore performance was lower on most of the
56 exotic species compared to the native species. We conclude that high chemical diversity and
57 large phytochemical uniqueness of the exotic species could be indicative of biological invasion
58 potential.

59

60

61

62

63

64

65

66

67

68

69 **Introduction**

70

71 Many plant species have been introduced to other continents either accidentally or by deliberate
72 introduction for, for example, horticultural purposes. Moreover, over the past few decades,
73 distributions of species have shifted pole-wards and will continue to do so under current and
74 future climate change (Parmesan & Yohe 2003; Walther 2010). The redistribution of species and
75 changing climatic conditions can lead to biological invasions whereby exotic species
76 increasingly dominate native ecosystems and alter various aspects of ecosystem functioning
77 (Thuiller *et al.* 2007; Vila *et al.* 2011).

78 There are many different hypotheses on why exotic plants may become invasive
79 (Catford *et al.* 2009). The enemy release hypothesis (Keane & Crawley 2002) assumes that the
80 loss of specialist natural enemies in the new range releases the plants from top-down control and
81 contributes to biological invasions. The evolution of increased competitive ability (EICA)
82 hypothesis (Blossey & Notzold 1995) predicts this leads to a decrease in chemical defenses and
83 increased competitive ability. The novel weapons hypothesis, on the other hand, poses that exotic
84 plants may have secondary compounds in their root exudates that are not found in native plants
85 which are toxic to unadapted native species (Callaway & Aschehoug 2000; Callaway &
86 Ridenour 2004). This hypothesis can be extended to shoot chemistry and its effect on
87 aboveground unadapted native herbivores or pathogens (Cappunicco & Arnason 2006; Barto *et*
88 *al.* 2010; Schaffner *et al.* 2011, Enge *et al.* 2012). A literature review revealed that invasive
89 exotic plants are indeed more likely to have unique secondary compounds that are not found in
90 non-invasive exotic plants and native plants, suggesting that novel chemistry can indeed
91 contribute to invasion success (Cappuccino & Arnason 2006). Most results cited in this review

92 were based on chemical analyses targeted towards specific (groups of) known defense
93 compounds. Thus far, experimental studies investigating the role of novel plant chemistry in
94 biological invasions focused on one or a few compound classes and/or one or a few plant species
95 (Callaway *et al.* 2008; Lankau *et al.* 2009; Barto *et al.* 2010; Enge *et al.* 2012; Kaur *et al.* 2012;
96 Whitehill *et al.* 2012; Qin *et al.* 2013; Svensson *et al.* 2013). One of the more broad studies to
97 date analyzed several phenolic compounds of nine native and nine invasive plant species (Kim &
98 Lee 2011). However, comprehensive chemical analytical techniques, e.g. untargeted
99 metabolomics, nowadays enable the simultaneous screening of several hundreds of known and
100 unknown plant metabolites belonging to different chemical classes that may be present in a
101 single plant species (Fiehn *et al.* 2000; Macel *et al.* 2010). Such an untargeted metabolomic
102 profiling or fingerprinting approach provides a much broader view of plant chemistry. Moreover,
103 because of the global extraction and analysis approach, it can be applied to any plant species.
104 Metabolomic profiling has, for example, been used to identify previously unknown plant defense
105 compounds in *Chrysanthemum* (Leiss *et al.* 2009) and *Brassica oleraceae* (Jansen *et al.* 2009).

106 Here we used a comprehensive untargeted metabolomics approach to investigate the
107 differences in shoot chemistry of a range of successful range expanding exotic plant species and
108 their native sister species of the same genus. We also tested the performance of a native
109 generalist herbivore (*Mamestra brassicae* L.) on the plants, and analyzed the effect of herbivore
110 damage on the metabolomics profiles. We expected that the exotic species would be
111 phytochemically unique, with compounds not found in the native plants (Cappuccino & Arnason
112 2006) and that herbivore performance would be lower on the exotic species. We also expected
113 the chemical profiles to change slightly after herbivore attack due to the possible induction of
114 defenses (Karban & Baldwin 1997). By investigating both uninduced and herbivory-induced

115 plants we could cover a wider range of the metabolome of the plants. Most studies on exotic
116 plant defenses only considered constitutive defense levels (but see Cipollini *et al.* 2005).

117 All selected plant species were of the Asteraceae family because we expected the
118 chemical defenses within one plant family to be more comparable than between families. Three
119 of the exotic species, *Senecio inaequidens*, *Solidago gigantea* and *Bidens frondosa* were
120 introduced in Europe from other continents and are among the most invasive terrestrial plants in
121 Western Europe (Lambdon *et al.* 2008). *Se. inaequidens* is known to contain moderate amounts
122 of (hepato)toxic pyrrolizidine alkaloids which are also found in native *Senecio* species (Cano *et*
123 *al.* 2009). Native snails readily fed on the exotic *Senecio* species, while a native specialist
124 herbivore that is adapted to the alkaloids did not survive on it (Macel *et al.* 2002; Cano *et al.*
125 2009). The latter suggests that other compounds besides pyrrolizidine alkaloids are playing a role
126 in herbivore resistance in the invasive *Senecio* species. *So. gigantea* is known to contain various
127 commonly occurring terpenoids (Hull-Sanders *et al.* 2009) and the chemistry of *B. frondosa* is
128 largely unknown thus far. The other three exotic species, *Artemisia biennis*, *Tragopogon dubius*
129 and *Tanacetum parthenium* are Eurasian plants native to South or South East Europe. They are
130 exotic in North West Europe where they have been increasing in abundance over the last 50
131 years (Tamis *et al.* 2005). An earlier study found that these range expanding plants are less
132 affected by herbivores, possibly due to higher total levels of phenolic compounds (Engelkes *et*
133 *al.* 2008). Similar to essential oils of their native congeners, extracts of both *A. biennis* and *T.*
134 *parthenium* contain a rich diversity of terpenoids that may have antibiotic or insecticidal
135 properties (Lopes-Lutz *et al.* 2008; Wolf *et al.* 2011). Other than this, little is known about the
136 defense chemistry of these range expanding exotic species. Plants were grown in the greenhouse
137 and received either no herbivory or herbivory by the generalist herbivore *M. brassicae*. LC-MS

138 metabolomics on the shoots was performed and larval weight of *M. brassicae* before and after
139 feeding on the plants was measured.

140

141 **Material and methods**

142

143 **Plant and herbivore species**

144 For each exotic plant species we chose a native relative from the same genus co-occurring with
145 the exotics in the invaded habitat (Table 1) so we could make a phylogenetically controlled
146 comparison. Not all the exotic species are considered highly invasive but they all have been
147 increasing in abundance in the Netherlands over the last 50 years (Tamis 2005). Three exotic
148 plant species originated from other continents, whereas three other exotic species were
149 intracontinental range expanders within Eurasia. All plants were grown from seed collected from
150 wild local Dutch populations by Dutch seed companies. Larvae of the cabbage moth, *Mamestra*
151 *brassicae* (Lepidoptera; Noctuidae), were obtained from a laboratory rearing at the Entomology
152 Department of Wageningen University, the Netherlands where they were reared on cabbage for
153 many generations. This native palearctic generalist herbivore feeds from plant of many different
154 families, including the Asteraceae (Theunissen *et al.* 1985). We used third instars, reared on
155 artificial diet, in the experiment.

156

157 **Experiment**

158 Seeds were surface sterilized with a 1% hypochlorite solution and germinated on glass beads
159 with demineralized water in a growth cabinet at 15-20°C, 8-16 hrs D/L. Two weeks after
160 germination, the seedlings were transferred to 1 L pots with unsterilized field soil collected from

161 the nature reserve Millingerwaard (51°87`N, 6°01`E). The pots were placed in a greenhouse with
162 conditions of 60% RH, 16 ±2°C-21±2° C, 8-16 hrs D/L in a randomized block design (5 blocks).
163 Ten to 20 plants were used of each species. After 8 weeks, defenses in half the plants were
164 induced by placing one *M. brassicae* third instar larva in a clip cages (Ø 8 cm) attached to one
165 leaf of each plant. Leaves of the same age were chosen within each plant species and control
166 plants received clip cages without herbivores. Larvae were weighed before they were placed on
167 the plants. Clip cages were moved to another leaf when the first leaf was almost defoliated. After
168 5 days of *M. brassicae* feeding, the larvae were removed and weighed again 5 hrs after removal.
169 Directly after the larvae were removed, all leaves younger than the leaf with the clip cage were
170 harvested and immersed in liquid nitrogen. Leaves were freeze dried and stored at -80°C until
171 further analysis.

172

173 **Untargeted metabolomics using LC-QTOF-MS**

174 Plant samples were analyzed for variation in semi-polar metabolite composition using an
175 untargeted accurate mass LC-MS approach, with on-line absorbance spectra measurements using
176 a photodiode array (PDA) detector, essentially as described in (De Vos *et al.* 2007). In short, 20
177 mg DW of frozen plant material was weighed in glass tubes and extracted with 2 ml of 75%
178 methanol in water containing 0.1% formic acid. Samples were sonicated for 15 minutes at 40
179 kHz and centrifuged, and then filtered (Captiva 0.45 µm PTFE filter plate, Ansys Technologies)
180 into 96-well plates with 700µl glass inserts (Waters) using a TECAN Genesis Workstation.
181 Extracts (5 µl) were injected using an Alliance 2795 HT instrument (Waters), separated on a
182 Phenomenex Luna C18 (2) column (2.0x 150 mm, 3 µm particle size) using a 45 minutes 5-75%
183 acetonitrile gradient in water (both acidified with 0.1% formic acid) and then detected firstly by a

184 photodiode array detector (Waters 2996) at a wavelength range of 220-600nm and secondly by a
185 Waters-Micromass QTOF Ultima MS with negative electrospray ionization at a mass range of
186 m/z 80-1500. Leucine enkaphalin was used as lock mass for on-line mass calibration.

187

188 **Data pre-processing and reduction of the dataset**

189 Metalign software (www.metalign.nl) was used to extract and align all accurate mass signals
190 (with signal to noise ratio ≥ 3) from the raw data files. To improve the quality of the data set,
191 signals present in at least 5 samples and at least in one an amplitude higher than 100 (about 5
192 times the noise value) were subsequently selected, resulting in a dataset of 15824 mass signals.
193 Finally, the so-called multivariate mass spectra reconstruction strategy (Tikunov et al. 2005) was
194 used to remove data redundancy by both retention time and sample-dependent clustering of
195 signals derived from the same compound, i.e. isotopes, adducts and in-source fragments. This
196 clustering of the 15824 mass signals revealed 1122 reconstructed metabolites and 896 (5.6%)
197 single, non-clustered, mass signals. From each reconstructed metabolite the signal intensity of
198 the most intense mass was selected for further statistical analyses. The LC-MS approach mainly
199 detects semi-polar non-volatile secondary metabolites from different biochemical pathways,
200 including phenolics, flavonoids, sesquiterpenes, alkaloids and saponins, as well as some primary
201 metabolites, such as organic acids and sugars. Both individual mass signals and reconstructed
202 metabolites, based on retention time dependent clustering of signals over samples (Tikunov *et al.*
203 2005), were taken into account.

204

205 **Data Analysis**

206

207 Seven quality control samples, consisting of a mixture of the methanol extractions of the 13 plant
208 species used in the experiment, were included in the LC-MS analysis. The error rate of mass
209 signal detection (type II error), calculated as $\text{error} = 1 - \text{fraction correct}^{1/n}$, in these seven control
210 samples was 0.07, which is comparable with other studies using this method (Vorst *et al.* 2005).
211 Statistical analyses were performed in R 2.11.1. Number of total mass signals and total number
212 of metabolites were analyzed with analysis of variance (ANOVA) with origin, herbivory
213 treatment and species nested within origin as fixed factors and the interaction between origin x
214 herbivory treatment included in the model. The number of unique mass signals and unique
215 reconstructed metabolites were not normally distributed and were rank-transformed and tested
216 with a multi-factorial ANOVA adjusted for ranks (Sokal & Rohlf 1995). Differences within
217 species pairs were tested with separate ANOVAs and significance levels were adjusted for
218 multiple tests with Bonferroni corrections (Sokal & Rolf 1995). The relative growth of the *M.*
219 *brassicae* larvae was calculated by end weight divided by begin weight of the larvae. The
220 relative growth data were square root transformed to meet the assumptions of normal distribution
221 and tested for differences between native and exotic species using ANOVA's with Bonferroni
222 corrections within the congeneric species pairs. Spearman rank correlations were used to test the
223 relation between *Mamestra* relative growth and number of metabolites.

224

225

226 **Results**

227

228 **Chemical diversity**

229

230 As a first step to compare the chemical diversity between the Asteraceae species, we analyzed
231 the overlap in metabolomic profiles. Our results indicate a high diversity in secondary chemistry
232 among the tested plant species. Overall, most of the detected mass signals (complete or
233 fragmented metabolites) were species specific (Fig. 1, black bars). Most of the 15824 individual
234 mass signals, 46.8%, occurred in single plant species. Only 2.6 % of all mass signals overlapped
235 and were found in all plant species. Similarity in mass signals between individual plants within a
236 species ranged from 13.3% for *Senecio inaequidens* to 57.5 % for *Tragopogon dubius*. This
237 means that there was considerable variation in chemical profiles within *Se. inaequidens* but
238 variation was much lower within the other species. The similarity in mass signals between all
239 plants within a genus ranged from 2.5 % in *Senecio* (three species) to 31 % in *Bidens*. The
240 frequency distribution of the reduced dataset, the reconstructed metabolites (cluster data),
241 showed a similar pattern albeit less pronounced (Fig.1; grey bars), 28 % of the 1122
242 reconstructed metabolites occurred in only a single species while 7.6 % of the metabolites were
243 shared among all species. These frequency distributions remained similar when the threshold
244 level was increased to ten times the noise level, which indicates that the observed distribution
245 was not due to small peaks that are close to the detection limit. One of the metabolites that was
246 present in all plants was chlorogenic acid (mass 353). This phenylpropanoid is commonly found
247 in plants, but is particularly abundant in the Asteraceae (Mølgaard & Ravn 1988).

248

249 **Native vs. exotic species**

250

251 The range-expanding exotic plant species contained, on average, a higher total number of
252 reconstructed metabolites than their native congeners (Fig. 2A, $P < 0.0001$, Table 2). The total

253 number of mass signals (complete or fragmented metabolites) showed a similar but non-
254 significant difference (Fig. 2B, $P = 0.78$). Furthermore, the exotic plants also contained more
255 species-unique metabolites than their native congeners (both mass signals and reconstructed
256 metabolites, Fig. 2C, D, $P < 0.005$, Table 2). The proportion of unique mass signals relative to
257 the total number was also higher in exotic plants (31%) than in natives (24%) (ANOVA on
258 ranks, $H=4.51$, $df=1$, $P < 0.05$). While there was an overall difference between native and exotic
259 species, the differences between genera in number of metabolites were considerable (Table 2,
260 Fig. 3). *Solidago* species, and specifically the exotic *So. gigantea*, accumulated relatively high
261 numbers of unique compounds, more than twice as much as the other species (Fig.3). On the
262 other hand, the exotic *Bidens frondosa* and its native congener *B. tripartita* shared all metabolites
263 with other species analyzed. When focusing on individual congeneric species pairs, in 5 out of
264 the 7 paired species comparisons the exotic species possessed more unique metabolites than the
265 native species (Fig.3).

266

267 **Herbivory**

268

269 Herbivory increased the number of metabolites in both native and exotic plant species to a
270 similar extent (3.8% and 2.5 % increase respectively, non-significant interaction between origin
271 and herbivory treatment), thus the overall pattern of exotic species having more unique chemical
272 compounds remained similar to that in the uninduced plants (Fig 2 grey bars, Table 2).

273 Interestingly, in 3 of the 4 pairs (of the total of 7 pairs) where the exotics have more unique
274 metabolites, the relative growth of the *M. brassicae* caterpillars was also significantly lower on
275 the exotic species (*Artemisia*, *Senecio*, *Solidago*). Overall, the caterpillars grew 3 to 10 times

276 faster on the native than on these three exotic species (Table 3). *Tragopogon* was the exception
277 to this pattern, as caterpillars grew significantly faster on the exotic species (Table 3). Overall,
278 there was no direct correlation between the number of unique metabolites and the relative growth
279 rate of the caterpillars (R_s 0.01, $P = 0.94$, $N = 112$ (cluster data per individual plant and
280 caterpillar) and R_s -0.14, $P = 0.63$, $N = 13$ (averages per species)). Total number of metabolites
281 was also not correlated with caterpillar relative growth (R_s -0.01, $P = 0.91$, $N = 112$).

282

283 **Discussion**

284

285 The results of our comparative metabolomics analyses of the different species showed that the
286 successful exotic species had more total and more unique metabolites than native congeners,
287 both in uninduced and herbivore induced plants. The exotic species were thus overall more
288 chemically diverse than the native species and also more phytochemically unique. Previous
289 studies using more targeted chemical analyses have shown that phytochemical uniqueness may
290 play a role in the invasion of exotic plants (e.g. Callaway & Aschehoug 2000, Cappuccino &
291 Arnason 2006, Kim & Lee 2011, Enge et al. 2012, Svenson et al. 2013). Organisms, e.g.
292 herbivores and competitors, in the new or introduced range may not be adapted to the unique
293 compounds that are new to the introduced range (e.g. Callaway & Aschehoug 2000, Callaway et
294 al. 2008, Schaffner et al. 2011, Enge et al. 2012, Svenson et al. 2013). Our results further showed
295 that metabolomes were highly species-specific, with most species containing unique metabolites
296 not found in other species. Both the native and the invasive species were therefore
297 phytochemically unique to some degree, although the proportion of unique metabolites was
298 higher in the exotic plants. Consequently, this indicates that in general any exotic plant species,

299 also non-invasive ones, is likely to have at least some metabolites that are new to organisms in
300 the introduced range. We cannot be absolutely certain that the unique compounds found here are
301 not present in any other species as we included only one or two native sister species in our study.
302 Nonetheless, the high proportion of unique metabolites of the successful exotic plants studied
303 here suggests that high chemical diversity and high phytochemical uniqueness may be indicative
304 of biological invasion potential. High chemical diversity and greater chemical uniqueness can be
305 beneficial in several ways. High diversity of plant secondary metabolites can lead to higher
306 resistance to for instance herbivory by impeding counter-adaptations by (native) herbivores and
307 by making the plant more toxic if compounds act synergistically. Furthermore, plants with high
308 chemical diversity may be resistant to a wider range of antagonists if individual metabolites act
309 specifically against different organisms (Berenbaum 1985; Jones & Firn 1991; Fritz & Simms
310 1992). Possibly most importantly, the high proportion of unique chemicals in exotic plant species
311 could increase the chance of having a potent compound or combinations of compounds to which
312 native species in the introduced range are not adapted yet.

313 In our metabolomic fingerprinting approach we did not attempt to identify all of the
314 metabolites because of the large number of unknown metabolites that were detected (95%).
315 Therefore, we cannot distinguish whether the exotic plants in this study contained completely
316 different classes of compounds compared to native species, or produced compounds that were
317 structurally related to compounds present in the native species. Although it may be more likely
318 that organisms in the introduced range of an exotic species are not adapted to a metabolite from a
319 class of compounds that is completely absent in the introduced range, small modifications of
320 structurally related compounds may already require new adaptations as they can have different
321 modes of action (Macel *et al.* 2005; van Leur *et al.* 2008). With the method we used we mostly

322 analyzed plant secondary metabolites, which generally have a function in the plant's interactions
323 with its biotic and abiotic environment (Fritz & Simms 1992). In how far the metabolites
324 analyzed in this study are used as defenses or offense (novel weapons) in the new range we
325 cannot say.

326 We included the exotic invaders from other continents and the exotic range expanders in
327 our study. In both groups of exotics, exotic plants had more unique metabolites than the natives
328 in two out of the three congeneric species pairs (Fig. 3). Invasion processes from exotic species
329 from other continents may be different from range expanding exotics, such as only partial enemy
330 release and continuing gene flow with source populations in the native range (Morrien et al.
331 2010). Nonetheless, high levels of chemical diversity and chemical uniqueness in individual
332 plants could be related to successful spread and/or invasion of exotic species, independent of
333 their origin. Furthermore, plants from lower latitudes are expected to have stronger defenses
334 against herbivory due to the greater herbivore pressure at lower latitudes compared to higher
335 latitudes (Bolser & Hay 1996; Pearse & Hipp 2012). Plants that are shifting to higher latitude
336 areas could therefore include highly defended plants. It is also possible that selection during
337 range expansion or invasion favors plants with a higher chemical diversity and chemical
338 uniqueness.

339 It would be interesting to see if the results that we found here would be similar on other
340 continents as well. Some of the native species in our study are invasive elsewhere, such as *Se.*
341 *jacobaea*, *Se. vulgaris*, *T. vulgare* and *A. vulgaris*. In the introduced range of an exotic species
342 intraspecific hybridization (admixture) can occur between populations that were isolated from
343 each other in the native range. Admixture is thought to play an important role in biological
344 invasions (Ellstrand & Schierenbeck 2006; Verhoeven *et al.* 2011). Benefits of admixture

345 include an increase of standing genetic variation, the formation of novel genotypes and lift of
346 inbreeding load. A recent study showed that outbreeding increases the number of phenolic
347 compounds in plants (Campbell *et al.* 2013). If outbreeding in general increases the number of
348 defense compounds in plants and intraspecific hybridization has occurred in the exotic species,
349 then it is possible that successful invasive admixed genotypes in the introduced range of a
350 species could have a higher number of defense compounds than plants in their native range. This
351 would be an additional explanation for the higher numbers of metabolites in the exotic plants.

352 The performance of the native generalist herbivore *M. brassicae* was significantly lower
353 on three of the six exotic species when compared to their native congeners (*Artemisia*, *Senecio*,
354 *Solidago*). This suggests that some, but not all, of the exotic species in our study were better
355 defended than native species against this native herbivore. The three exotic species on which *M.*
356 *brassicae* performed poorly also contained significantly higher number of metabolites than the
357 native sister plants. However, we did not find a direct linear correlation between herbivore
358 performance and number of metabolites among all the species. Possibly only a few metabolites
359 or a combination of active compounds are responsible for the low performance of this particular
360 herbivore (van Leur *et al.* 2008). Larval performance on the exotic range expanding *Tragopogon*
361 species was higher compared to performance on the native *Tragopogon*, even though the exotic
362 species contained a higher number of metabolites. We expected that generally herbivore
363 performance on the exotic plants would be lower but here we found that does not hold for all the
364 exotic species we tested. Indeed there is quite some variation in the results obtained with
365 manipulative herbivore experiments in which native and exotic congeners are compared. For
366 example, it was shown that generalist snails fed more on the exotic *Se. inaequidens* than on the
367 native *Se. vulgaris* (Cano *et al.* 2009). In an early study on range expanding exotic plants,

368 generalist locusts were performing worse on the exotics, while generalist aphids performed
369 equally well on both exotics and natives (Engelkes *et al.* 2008). The palatability of exotic species
370 thus also depends on which native generalist herbivore species is tested.

371 In conclusion, our untargeted metabolomics study showed that successful exotic plant
372 species had a higher diversity of metabolites and more unique metabolites compared to
373 congeneric native species. This pattern was found for classic invaders from other continents as
374 well as for plants that are currently successfully expanding their range on the same continent. In
375 addition to one single highly potent novel compound, high chemical diversity and phytochemical
376 uniqueness of many compounds may thus also be indicative of plant species invasiveness.
377 Furthermore, combinations of compounds acting in synchrony are likely to be important. The
378 exact function of the high chemical diversity and uniqueness in exotic plants and its role in plant
379 invasions still needs further testing. Whether this high diversity is due to post-introduction
380 evolution or is a pre-existing trait of invasive exotic plants also remains to be elucidated.

381

382 **Acknowledgements**

383

384 We thank B. Schipper and H. Jonker for technical assistance; J. Harvey for providing *Mamestra*
385 larvae; T. Engelkes, E. Morriën and A. Meisner for providing seed material, and three
386 anonymous reviewers for helpful comments on earlier versions of the manuscript. This work was
387 supported by a NWO-VICI grant to WHvdP. RCHdV was supported by the Centre for
388 BioSystems Genomics, which is part of the Netherlands Genomics Initiative / Netherlands
389 Organization for Scientific Research.

390

391

392 **References**

- 393 1.
394 Barto, E.K., Powell, J.R. & Cipollini, D. (2010). How novel are the chemical weapons of garlic
395 mustard in North American forest understories? *Biol. Invasions*, 12, 3465-3471.
- 396 2.
397 Berenbaum, M.R. (1985). Brenton revisited: interactions among allelochemicals in plants.
398 *Rec. Adv. Phytochem.*, 19, 139-169
- 399 3.
400 Blossey, B. & Notzold, R. (1995). Evolution of increased competitive ability in invasive
401 nonindigenous plants - a hypothesis. *J. Ecol.*, 83, 887-889.
- 402 4.
403 Bolser, R.C. & Hay, M.E. (1996). Are tropical plants better defended? Palatability and defenses
404 of temperate vs tropical seaweeds. *Ecology*, 77, 2269-2286.
- 405 5.
406 Callaway, R.M. & Aschehoug, E.T. (2000). Invasive plants versus their new and old neighbors:
407 A mechanism for exotic invasion. *Science*, 290, 521-523.
- 408 6.
409 Callaway, R.M., Cipollini, D., Barto, K., Thelen, G.C., Hallett, S.G., Prati D., *et al.* (2008).
410 Novel weapons: Invasive plant suppresses fungal mutualists in America but not in its
411 native Europe. *Ecology*, 89, 1043-1055.
- 412 7.
413 Callaway, R.M. & Ridenour, W.M. (2004). Novel weapons: invasive success and the evolution
414 of increased competitive ability. *Front. Ecol. Environ.*, 2, 436-443.
- 415 8.
416 Campbell, S.A., Thaler, J.S. & Kessler, A. (2013). Plant chemistry underlies herbivore-mediated
417 inbreeding depression in nature. *Ecol. Lett.*, 16, 252-260.
- 418 9.
419 Cano, L., Escarre, J., Vrieling, K. & Sans, F.X. (2009). Palatability to a generalist herbivore,
420 defence and growth of invasive and native *Senecio* species: testing the evolution of
421 increased competitive ability hypothesis. *Oecologia*, 159, 95-106.
- 422 10.
423 Cappuccino, N. & Arnason, J.T. (2006). Novel chemistry of invasive exotic plants. *Biol. Lett.*, 2,
424 189-193.
- 425 11.
426 Catford, J.A., Jansson, R. & Nilsson, C. (2009). Reducing redundancy in invasion ecology by
427 integrating hypotheses into a single theoretical framework. *Divers. Distrib.*, 15, 22-40.
- 428 12.
429 Cipollini, D., Mbagwu, J., Barto, K., Hillstrom, C. & Enright, S. (2005). Expression of
430 constitutive and inducible chemical defenses in native and invasive populations of
431 *Alliaria petiolata*. *J. Chem. Ecol.*, 31, 1255-1267.
- 432 13.

433 De Vos, R.C.H., Moco, S., Lommen, A., Keurentjes, J.J.B., Bino, R.J. & Hall, R.D. (2007).
434 Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass
435 spectrometry. *Nat. Protoc.*, 2, 778-791.

436 14.

437 Ellstrand, N.C. & Schierenbeck, K.A. (2006). Hybridization as a stimulus for the evolution of
438 invasiveness in plants? *Euphytica*, 148, 35-46.

439 15.

440 Enge, S., Nylund, G.M., Harder, T. & Pavia, H. (2012). An exotic chemical weapon explains low
441 herbivore damage in an invasive alga. *Ecology*, 93, 2736-2745.

442 16.

443 Engelkes, T., Morrien, E., Verhoeven, K.J.F., Bezemer, T.M., Biere, A., Harvey, J.A., *et al.*
444 (2008). Successful range-expanding plants experience less above-ground and below-
445 ground enemy impact. *Nature*, 456, 946-948.

446 17.

447 Fiehn, O., Kopka, J., Dormann, P., Altmann, T., Trethewey, R.N. & Willmitzer, L. (2000).
448 Metabolite profiling for plant functional genomics. *Nat. Biotechnol.*, 18, 1157-1161.

449 18.

450 Fritz, R.S. & Simms E.L. (eds) (1992). Plant Resistance to herbivores and pathogens – ecology,
451 evolution and genetics. University of Chicago Press, Chicago and London.

452 19.

453 Hull-Sanders, H.M., Johnson, R.H., Owen, H.A. & Meyer, G.A. (2009). Effects of polyploidy on
454 secondary chemistry, physiology, and performance of native and invasive genotypes of
455 *Solidago gigantea* (Asteraceae). *Am. J. Bot.*, 96, 762-770.

456 20.

457 Jansen, J.J., Allwood, J.W., Marsden-Edwards, E., van der Putten, W.H., Goodacre, R. & van
458 Dam, N.M. (2009). Metabolomic analysis of the interaction between plants and
459 herbivores. *Metabolomics*, 5, 150-161.

460 21.

461 Jones, C.G. & Firn, R.D. (1991). On the evolution of plant secondary chemical diversity. *Phil.*
462 *Trans. R. Soc. Lond. B*, 333, 273-280.

463 22.

464 Karban, R. & Baldwin, I.T. (1997). *Induced responses to herbivory*. The University of Chicago
465 Press, Chicago, U.S.A.

466 23.

467 Kaur, R., Gonzales, W.L., Llambi, L.D., Soriano, P.J., Callaway, R.M., Rout, M.E., *et al.* (2012).
468 Community impacts of *Prosopis juliflora* invasion: biogeographic and congeneric
469 comparisons. *Plos One*, 7, e44966

470 24.

471 Keane, R.M. & Crawley, M.J. (2002). Exotic plant invasions and the enemy release hypothesis.
472 *Trends Ecol. Evol.*, 17, 164-170.

473 25.

474 Kim, Y.O. & Lee, E.J. (2011). Comparison of phenolic compounds and the effects of invasive
475 and native species in East Asia: support for the novel weapons hypothesis. *Ecol. Res.*, 26,
476 87-94.

- 479 26.
480 Lambdon, P.W., Pysek, P., Basnou, C., Hejda, M., Arianoutsou, M., Essl, F., *et al.* (2008). Alien
481 flora of Europe: species diversity, temporal trends, geographical patterns and research
482 needs. *Preslia*, 80, 101-149.
- 483 27.
484 Lankau, R.A., Nuzzo, V., Spyreas, G. & Davis, A.S. (2009). Evolutionary limits ameliorate the
485 negative impact of an invasive plant. *Proc. Natl. Acad. Sci. U. S. A.*, 106, 15362-15367.
- 486 28.
487 Leiss, K.A., Maltese, F., Choi, Y.H., Verpoorte, R. & Klinkhamer, P.G.L. (2009). Identification
488 of chlorogenic acid as a resistance factor for thrips in *Chrysanthemum*. *Plant Physiol.*,
489 150, 1567-1575.
- 490 29.
491 Lopes-Lutz, D., Alviano, D.S., Alviano, C.S. & Kolodziejczyk, P.P. (2008). Screening of
492 chemical composition, antimicrobial and antioxidant activities of *Artemisia* essential oils.
493 *Phytochem.*, 69, 1732-1738.
- 494 30.
495 Macel, M., Bruinsma, M., Dijkstra, S.M., Ooijendijk, T., Niemeyer, H.M. & Klinkhamer, P.G.L.
496 (2005). Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore
497 species. *J. Chem. Ecol.*, 31, 1493-1508.
- 498 31.
499 Macel, M., Klinkhamer, P.G.L., Vrieling, K. & van der Meijden, E. (2002). Diversity of
500 pyrrolizidine alkaloids in *Senecio* species does not affect the specialist herbivore *Tyria*
501 *jacobaeae*. *Oecologia*, 133, 541-550.
- 502 32.
503 Macel, M., van Dam, N.M. & Keurentjes, J.J.B. (2010). Metabolomics: the chemistry between
504 ecology and genetics. *Mol. Ecol. Res.*, 10, 583-593.
- 505 33.
506 Mølgaard, P. & Ravn, H. (1988). Evolutionary aspects of caffeoyl ester distribution In
507 Dicotyledons. *Phytochem.*, 27, 2411-2421.
- 508 34
509 Morrien, E., Engelkes, T., Macel, M., Meisner, A., Van der Putten, W.H. (2010). Climate change
510 and invasion by intracontinental range-expanding exotic plants: the role of biotic
511 interactions. *Ann. Bot.* 105, 843-848.
- 512 35.
513 Parmesan, C. & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts
514 across natural systems. *Nature*, 421, 37-42.
- 515 36.
516 Pearse, I.S. & Hipp, A.L. (2012). Global patterns of leaf defenses in oak species. *Evolution*, 66,
517 2272-2286.
- 518 37.
519 Qin, R.M., Zheng, Y.L., Valiente-Banuet, A., Callaway, R.M., Barclay, G.F., Pereyra, C.S., *et al.*
520 (2013). The evolution of increased competitive ability, innate competitive advantages,
521 and novel biochemical weapons act in concert for a tropical invader. *New Phytol.*, 197,
522 979-988.
- 523 38.

524 Schaffner, U., Ridenour, W.M., Wolf, V.C., Bassett, T., Müller, C., Müller-Schärer, H., *et al.*
525 (2011). Plant invasions, generalist herbivores, and novel defense weapons. *Ecology*, 92,
526 829-835.

527 39.

528 Sokal, R.R. & Rohlf, F.J. (1995). *Biometry: the principals and practice of statistics in biological*
529 *research*. W.H. Freeman and Company, New York, U.S.A..

530 40.

531 Svensson, J.R., Nylund, G.M., Cervin, G., Toth, G.B. & Pavia, H. (2013). Novel chemical
532 weapon of an exotic macroalga inhibits recruitment of native competitors in the invaded
533 range. *J. Ecol.*, 101, 140-148.

534 41.

535 Tamis, W.L.M. (2005). Changes in the flora of the Netherlands in the 20th century. *Gorteria*,
536 Supplement 6, 154-218.

537 42.

538 Tamis, W.L.M., Van't Zelfde, M., Van der Meijden, R. & De Haes, H.A.U. (2005). Changes in
539 vascular plant biodiversity in the Netherlands in the 20th century explained by their
540 climatic and other environmental characteristics. *Clim. Change*, 72, 37-56.

541 43.

542 Theunissen, J., de Ouden, H. & Wit, A.K.H. (1985). Feeding capacity of caterpillars on cabbage,
543 a factor in crop loss. *Entomol. Exp. Appl.*, 39, 255-260.

544 44.

545 Thuiller, W., Richardson, D.M. & Midgley, G.F. (2007). Will climate change promote invasive
546 species? In: *Ecological Studies* (ed. Nentwig, N.). Springer Verlag Berlin, Germany, pp.
547 197-211

548 45.

549 Tikunov, Y., Lommen, A., de Vos, C.H.R., Verhoeven, H.A., Bino, R.J., Hall, R.D., *et al.*
550 (2005). A novel approach for nontargeted data analysis for metabolomics. Large-scale
551 profiling of tomato fruit volatiles. *Plant Physiol.*, 139, 1125-1137.

552 46.

553 van Leur, H., Vet, L.E.M., Van der Putten, W.H. & van Dam, N.M. (2008). *Barbarea vulgaris*
554 glucosinolate phenotypes differentially affect performance and preference of two
555 different species of lepidopteran herbivores. *J. Chem. Ecol.*, 34, 121-131.

556 47.

557 Verhoeven, K.J.F., Macel, M., Wolfe, L.M. & Biere, A. (2011). Population admixture, biological
558 invasions and the balance between local adaptation and inbreeding depression. *Proc. R.*
559 *Soc. B-Biol. Sci.*, 278, 2-8.

560 48.

561 Vila, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarosik, V., Maron, J.L., *et al.* (2011). Ecological
562 impacts of invasive alien plants: a meta-analysis of their effects on species, communities
563 and ecosystems. *Ecol. Let.*, 14, 702-708.

564 49.

565 Vorst, O., de Vos, C.H.R., Lommen, A., Staps, R.V., Visser, R.G.F., Bino, R.J., *et al.* (2005). A
566 non-directed approach to the differential analysis of multiple LC-MS-derived metabolic
567 profiles. *Metabolomics*, 1, 169-180.

568 50.

569 Walther, G.R. (2010). Community and ecosystem responses to recent climate change. *Phil.*
570 *Trans. R. Soc. B.*, 365, 2019-2024.
571 51.

572 Whitehill, J.G.A., Opiyo, S.O., Koch, J.L., Herms, D.A., Cipollini, D.F. & Bonello, P. (2012).
573 Interspecific comparison of constitutive ash phloem phenolic chemistry reveals
574 compounds unique to manchurian ash, a species resistant to emerald ash borer. *J. Chem.*
575 *Ecol.*, 38, 499-511.
576 52.

577 Wolf V.C., Berger U., Gassmann A. & Müller C. (2011). High chemical diversity of a plant
578 species is accompanied by increased chemical defence in invasive populations. *Biol.*
579 *Invasions*, 13, 2091-2102.
580
581

582

583

584

585

586

587

588

589

590

591

592

593

594

595

596 **Table 1.** Origin of species used in the experiment. Underlined species names indicate exotic
 597 species. Some exotic plants originate in Eurasia and are non-native to the Netherlands, others
 598 originate from other continents.

Plant Species	Origin	Dutch population used in experiment	Present since
<u>Artemisia biennis</u>	Eurasia	Dodewaard	1950
<i>Artemisia vulgaris</i>	Eurasia	Gendtse Polder	Native
<u>Bidens frondosa</u>	North America	Polder Zeevang	1900
<i>Bidens tripartite</i>	Eurasia	Polder Zeevang	Native
<u>Senecio inaequidens</u>	South Africa	Millingerwaard	1925
<i>Senecio vulgaris</i>	Eurasia	Heerlen	Native
<i>Senecio jacobaea</i>	Eurasia	Millingerwaard / Meijendel	Native
<u>Solidago gigantean</u>	North America	Gendtse Polder	1900
<i>Solidago virgaurea</i>	Eurasia	Seed company	Native
<u>Tanacetum parthenium</u>	Eurasia	Seed company	1500
<i>Tanacetum vulgare</i>	Eurasia	Seed company	Native
<u>Tragopogon dubius</u>	Eurasia	Amersfoort	1925
<i>Tragopogon pratensis</i>	Eurasia	Ooijpolder	Native

599

600 **Table 2.** Effect of plant origin, species and herbivory treatment on the number of LC-MS mass
 601 signals and reconstructed metabolites in plants. Table entries are *F*-values of multi-factorial
 602 ANOVA. *N*=227

	d.f.	Mass signals		Reconstructed metabolites	
		Total	Unique ^a	Total	Unique ^a
Origin	1	0.76	28.52 ***	69.26***	15.54**
Species within Origin	11	74.69***	189.60***	152.68***	203.54***
Treatment	1	10.34**	6.77 *	9.54 **	2.11
Origin x Treatment	1	0.14	0.53	0.87	0.17

603 *** *P* < 0.0001, ** *P* < 0.005, * *P* < 0.05. ^a rank transformed data

604
 605
 606
 607
 608
 609
 610
 611
 612
 613
 614
 615
 616

617 **Table 3.** Relative growth of *Mamestra brassicae* larvae on exotic and native plants. Relative
 618 growth was calculated as end weight / begin weight. * indicates significant differences between
 619 exotic and native species within congeneric species pairs (ANOVA, Significance level after
 620 Bonferroni correction for multiple tests * P < 0.008, + P = 0.05).

Genus	Species	Origin	Mean growth (\pm SE)	N
<i>Artemisia</i>	<i>biennis</i>	exotic	0.30 (\pm 0.12)	9
	<i>vulgaris</i>	native	2.96 (\pm 0.67)*	10
<i>Bidens</i>	<i>frondosa</i>	exotic	2.70 (\pm 0.85)	5
	<i>tripartita</i>	native	1.36 (\pm 0.63)	4
<i>Senecio</i>	<i>inaequidens</i>	exotic	1.33 (\pm 0.23)	10
	<i>jacobaea</i>	native	3.50 (\pm 0.78) +	10
	<i>vulgaris</i>	native	4.30 (\pm 0.67)*	10
<i>Solidago</i>	<i>gigantea</i>	exotic	0.72 (\pm 0.31)	10
	<i>virgaurea</i>	native	4.31 (\pm 0.79)*	10
<i>Tanacetum</i>	<i>parthenium</i>	exotic	0.85 (\pm 0.20)	9
	<i>vulgare</i>	native	1.63 (\pm 0.47)	9
<i>Tragopogon</i>	<i>dubius</i>	exotic	1.44 (\pm 0.31)	6
	<i>pratensis</i>	native	0.35 (\pm 0.10)*	10

621

622 **Figure legends**

623

624 **Figure 1** Diversity of metabolites in the 13 analyzed Asteraceae species. Frequency distribution
625 of the number of plant species each mass signal (black bars) and reconstructed metabolites (grey
626 bars) was detected in.

627

628 **Figure 2** Number of metabolites in native vs. invasive plants. Average total number of mass
629 signals (A) and number of reconstructed metabolites (B), number of species-unique masses (C)
630 and species-unique reconstructed metabolites (D) of exotic plants and native congeneric species.
631 Induced plants (gray bars) received herbivory by *Mamestra brassicae* caterpillars. Control plants
632 (black bars) were without herbivory. Plant origin differed significantly for total number of
633 reconstructed metabolites, and unique number of mass signals and reconstructed metabolites (**
634 $P < 0.005$, *** $P < 0.0001$, Table 2). Herbivory induced the total number of mass signals and
635 reconstructed metabolites in both native and exotic plants ($P < 0.05$, Table 2). Error bars indicate
636 standard errors of mean.

637

638 **Figure 3** Species-unique metabolites in native vs. invasive plants per genus (*Artemisia*, *Bidens*,
639 *Senecio*, *Solidago*, *Tanacetum*, *Tragopogon*). Average number (+SE) of unique metabolites per
640 species in native and invasive plants in the control treatment without herbivory. Grey bars
641 indicate exotic species, white bars indicate native species. Asterisks indicate significant
642 differences between exotic and native species within the same genus (ANOVA, all $P < 0.001$).

643