Erschienen in: Annals of Botany ; 130 (2022), 6. - S. 917-926
https://dx.doi.org/10.1093/aob/mcac127

Competition induces negative conspecific allelopathic effects on seedling recruitment

Ling Yuan[1,2], Junmin Li[1,2] and Mark van Kleunen[1,2,3,*]

[1]School of Advanced Study, Taizhou University, Taizhou 318000, Zhejiang, China, [2]Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Taizhou 318000, China and [3]Ecology, Department of Biology, University of Konstanz, D-78464 Konstanz, Germany
**For correspondence. E-mail mark.vankleunen@uni-konstanz.de*

• **Background and Aims** Some plant species suppress competitors through release of chemical compounds into the environment. As the production of allelochemicals may be costly, it would be beneficial if their production would only be induced when plants experience competition. We tested whether two plant species that frequently co-occur show evidence for induced allelopathy in response to intra- and interspecific competition.
• **Methods** We used the annual forb *Crepidiastrum sonchifolium* and the perennial forb *Achyranthes bidentata*, which are native to China and predominantly occur in ruderal communities, as focal species. We first grew the species without competition, with intraspecific competition and in competition with each other. We chemically analysed aqueous extracts made from these plants to test for evidence that the competition treatments affected the metabolomic profiles of the species. We then tested the effects of the aqueous extracts on seed germination and seedling growth of both plant species.
• **Key Results** Metabolomic analysis revealed that competition treatments modified the chemical profiles of the two study species. The root lengths of *A. bidentata* and *C. sonchifolium* seedlings were reduced by the aqueous plant extracts. For seedling root length of *A. bidentata*, heterospecific allelopathy was more negative than conspecific allelopathy, but for germination of *C. sonchifolium* seeds, the reverse was true. Moreover, conspecific allelopathic effects on germination of *A. bidentata* seeds and on seedling root length of both species were most negative when the aqueous extracts were made from plants that had experienced competition. In the case of seedling root length of *A. bidentata*, this effect was most negative when the plants had experienced interspecific instead of intraspecific competition.
• **Conclusions** We showed that plants change their metabolomic profiles in response to competition, and that this correlated with allelopathic inhibition of conspecific seed germination and seedling growth. We suggest that autoallelopathy for seed germination could function as a mechanism to avoid strong competition by keeping the seeds in a dormant state.

Key words: Allelochemicals, autoallelopathy, biochemical recognition hypothesis, metabolome, seedling emergence, self-allelopathy.

INTRODUCTION

Competition is fundamental to community assembly (Gause, 1934), evolution (Hutchinson, 1959) and ecosystem functioning (Tilman *et al.*, 2014). In general, nutrients, water and light are the three main resources that limit plant growth and for which plants compete. Competition between plants for common resources is considered to be mainly exploitative (i.e. they attempt to pre-empt resources before their neighbours do; Aschehoug *et al.*, 2016). For animals, however, it is well known that they can also directly fight for resources, including food, territory and mates. Such interference competition by physical means is not possible in plants. Nevertheless, some plants show interference competition by means of allelopathy, i.e. the suppression of competitors through release of chemical compounds into the environment (Callaway and Aschehoug, 2000; Duke, 2010; Hierro and Callaway, 2021).

These allelopathic compounds, released as root exudates, volatile organic compounds, leaf leachates or leaf litter, can suppress neighbouring plants directly or indirectly by impacting soil micro-organisms (Roberts and Anderson, 2001; Inderjit *et al.*, 2011, 2021). The benefit of inhibiting neighbours through increased allelopathy is expected to increase when the competition for limited resources becomes intense (Song *et al.*, 2008). As the benefits of allelopathy might depend on the environment, it could be that some allelochemicals are, just like defence chemicals against herbivores and pathogens, induced in response to environmental cues (Song *et al.*, 2008).

Although there has been a strong interest in the evolution of allelopathy, especially in invasive plant species (Callaway and Ridenour 2004), relatively few studies on allelopathy have explored its inducibility. Some studies reported that abiotic conditions (e.g. light and water availability) can plastically change the production of allelopathic compounds (Dayan, 2006; Kong *et*

al., 2006; Song *et al.*, 2008; Kato-Noguchi, 2011). Other studies showed that plants exposed to insect herbivory had increased allelopathic effects (Thelen *et al.*, 2005; Karban, 2007). However, studies that tested the direct effect of competition on allelopathy are limited. Lankau and Kleibenstein (2009) showed that sinigrin production in *Brassica nigra* was increased by a combination of herbivory and competition. Also, *Oryza sativa* grown with competitors exuded more allelochemicals than when grown alone (Kong *et al.*, 2006; Song *et al.*, 2008; Kato-Noguchi, 2011). Furthermore, Uesugi *et al.* (2019) provided evidence that interspecific competition increased allelopathy of an alien invasive plant (*Solidago altissima*), but that the benefit of such plasticity may vary across time and space. So, although some previous studies suggest that the presence of competitors might increase allelopathy, more studies are needed to test its generality.

It has been suggested that allopatric species should have stronger phytotoxic effects on each other than sympatric species, because the latter might have adapted to each other's chemicals during coevolution (Rabotnov, 1982). This idea is also the basis for the novel weapons hypothesis, which poses that invasive alien species have stronger negative allelopathic effects on species in their new range than on species in their native range (Callaway and Ridenour, 2004). On the other hand, the biochemical recognition hypothesis poses that seeds might use plant metabolites in the environment to eavesdrop on which species are present (Renne *et al.*, 2004, 2014). If there is a strong competitor present, the resident species might use the chemical cues to delay germination. If that hypothesis holds true, sympatric species might actually have stronger allelopathic effects on germination than would be the case for allopatric species. Moreover, as intraspecific competition is usually stronger than heterospecific competition (Adler *et al.*, 2018), the biochemical recognition hypothesis could also explain the occurrence of autoallelopathy (i.e. allelopathic effects of conspecific plants). However, it remains unknown whether changes in allelopathy in response to interspecific competition differ from changes in response to intraspecific competition.

Here we test whether two sympatric species native to China, the perennial forb *Achyranthes bidentata* and the annual forb *Crepidiastrum sonchifolium*, show evidence for induced allelopathy in response to intra- and interspecific competition. We first grew each species without competition, with intraspecific competition and in competition with each other, and produced aqueous extracts of those plants. We chemically analysed the extracts to test whether the competition treatments affected the metabolomic profiles of the species. Then to test whether the plants differed in their allelopathic activities, we tested the effects of aqueous extracts of these plants on seed germination and seedling growth of both plant species. We addressed the following questions. (1) Do aqueous extracts made from plants grown with competition have a stronger inhibitory effect on seed germination and seedling growth than extracts made from competition-free plants? (2) Do aqueous extracts made from plants grown with interspecific competition have a stronger inhibitory effect on germination and seedling growth of the other species than extracts made from plants grown with intraspecific competition? (3) Do the competition treatments affect the metabolomic profiles of the plants, and in turn affect germination and seedling growth?

MATERIALS AND METHODS

Study species

As study species, we used the annual forb *Crepidiastrum sonchifolium* (Maxim.) Pak & Kawano (Asteraceae) and the perennial forb *Achyranthes bidentata* Blume (Ameranthaceae). We chose those two species because they usually have high germination rates, and because a previous experiment showed that they can have allelopathic effects on each other (Yuan *et al.*, 2021). The species are native to eastern China, where they co-occur across a wide range of habitats, especially in ruderal habitats (Zhang and Ding, 1993). Seeds of the two species were obtained from commercial seed companies (*A. bidentata* from Green Forest Flower Seed Industry, Jiangsu Suqian, China; *C. sonchifolium* from Thousand Green Seed Company, Jiangsu Suqian, China).

Pre-cultivation of plants and production of aqueous extracts

For the production of aqueous extracts of the two study species, we first exposed plants to different competition treatments. On 29 March 2021, seeds of each of the two species were sown in plastic boxes (l × w × h: 54 × 28 × 5 cm) filled with a 1:1 (v:v) mixture of sand and vermiculite (both purchased from Xiaoxuan Horticulture, Hebei province, China). The boxes were placed in a phytochamber (daytime temperature, 18–21 °C; night-time temperature, 16–20 °C; daylength, 14 h; relative humidity, 60 %) at Taizhou University, China. Two weeks after emergence, the plants were transplanted into 2 L plastic pots filled with a 1:1 (v:v) mixture of sand and vermiculite. For each species, we had three treatments: competition-free (one plant per pot), intraspecific competition (two plants of the same species per pot) and interspecific competition (one plant of each species per pot). For each competition-free, intraspecific competition and interspecific competition treatment, we had ten pots. All 50 pots were then transferred to a greenhouse (daytime temperature, 22–25° C; night-time temperature, 18–21° C, daylength, 14 h; relative humidity, 60 %). The seedlings were fertilized once every 15 d during a period of 60 d with a Woshibao® liquid fertilizer (N, 50 g L^{-1}; P, 30 g L^{-1}; K, 50 g L^{-1}; Mg, 1.8 g L^{-1}; S, 2.2 g L^{-1}; micronutrients, 0.2 g L^{-1}; Woshibao Fertilizer Sales Co., Ltd, Lu'an, China). The fertilizer was diluted 1:200 with distilled water, and each pot received 100 mL each time. We watered the plants once a week or, when necessary, more frequently.

To produce aqueous extracts, we harvested, 60 d after the start of the pre-cultivation, for each of the two study species ten separate individual plants from each of the three competition treatments. For the intraspecific competition treatment, we used only one of the two plants per pot. We washed the roots free of growth medium, and then chopped each individual plant separately into pieces of 2 cm, and weighed them. For each plant, we mixed its shoot and root pieces, and we then took a random sample of 30 g. We determined the fresh weight of the remaining pieces, dried them for 48 h at 70 °C and weighed them again. Then, based on the total fresh weight and the fresh and dry weights of the remaining parts, we calculated the dry weight of the total plant. We also did this for the remaining

plants in the pots. We then transferred the sampled plant tissue into beakers containing 90 mL of distilled water and let it soak for 24 h at room temperature. This was done for each of the individual plants separately. Thereafter, the extracts were filtered, first through a layer of Whatman No. 1 filter paper, and then – to remove fungal spores – through a 0.8 µm filter membrane (25 mm in diameter) into separate autoclaved Falcon tubes. A fresh filter membrane was used each time. For each of the 60 resulting filtrates, we filled two Falcon tubes, which were stored at –20 °C until use.

Metabolomic analysis of the plant extracts

To assess whether the two species differed in their phytochemical contents, and whether treatments altered them, for each of the six species × competition treatment combinations, the metabolome of five samples (i.e. individual plants) were analysed. The analyses of the 30 samples were done by Wuhan Metware Biotechnology Co., Ltd (Wuhan, China).

After thawing the 30 samples, we mixed each sample separately with a vortex for 10 s, and transferred 3 mL into a 10 mL centrifuge tube. We then immersed the sample in liquid nitrogen and freeze-dried it in a lyophilizer. Thereafter, we added 100 µL of 70 % methanol internal standard extract. We vortexed each sample for 3 min, and then centrifuged (12 000 rpm, 4 °C) it for 10 min. The supernatant was filtered through a microporous filter membrane (0.22 µm) and then stored in a sample flask for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. The samples were analysed on a UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/; MS, Applied Biosystems 4500 Q TRAP, https://www.thermofisher.cn/cn/zh/home/brands/applied-biosystems.html) according to the protocols of Wuhan Metware Biotechnology Co., Ltd, with some modifications (details are provided in Supplementary data Appendix S1).

Experimental set-up

To test for potential allelopathic effects of the aqueous extracts of each species on their germination and seedling growth, we set up a Petri dish experiment. We filled 380 Petri dishes (6 cm in diameter) with agar gel into which we had mixed the aqueous plant extracts. The agar gel was made of 12 g high-strength agar with 30 g of sucrose and 3.225 g of Murashige and Skoog culture medium in 1 L of distilled water. We adjusted the pH of the agar solution to 6.0 with NaOH and HCl. We poured the agar medium into 1 L glass bottles and autoclaved it for 15 min at 120 °C and a pressure of 100 kPa. After autoclaving, the hot agar medium was placed into a 40 °C water bath for 10 min to cool down, while staying fluid. We thawed the 60 aqueous plant extracts in a heating cabinet at 35 °C for 30 min. The extracts were filtered once more through 0.8 µm filter membranes, and thereafter 10 mL of a 1:2 (v:v) mixture of plant leachates and agar medium was poured into each Petri dish. For each of the 60 extracts, we filled six Petri dishes (three for each test species). As control treatment, we filled 20 Petri dishes with agar without plant extract (resulting in a total of 380 Petri dishes). As the osmolality of the medium might affect seed germination (Inderjit and Nilsen, 2003; Oduor *et al.*, 2020), we measured osomolality of each of the 60 extracts three times with an osmometer (Wescor 5600, Shanghai Pengqi Scientific Instrument Co., Ltd). This was done after filtration of the extracts through the 0.8 µm syringe filters. For the control agar solution without plant extracts, we adjusted the osmolality to the mean value calculated across all 60 plant extracts (mean ± s.e. = 0.0219 ± 0.0002 Osmol kg^{-1}) by mixing the agar with a polyethylene glycol (PEG) 8000 (Sigma-Aldrich, Steinheim, Germany) solution, which had a concentration of 0.041 g PEG mL^{-1}.

On 12 August 2021, we placed seeds of *A. bidentata* and *C. sonchifolium* into separate Petri dishes. Before sowing, we had sterilized the seeds for 5 min in a 5 % sodium hypochlorite solution, after which we rinsed them with distilled water. In each Petri dish, we placed ten seeds of one of the two species. For each of the 60 plant extracts (2 allelopath species × 3 competition treatments × 10 replicate pots), we had three replicate Petri dishes per test species. In addition, we had ten replicate Petri dishes for the PEG control per test species. The Petri dishes were sealed with parafilm to avoid loss of water, and were randomly allocated to positions in a phytochamber (daytime temperature, 21 °C; night-time temperature, 18 °C; daylength, 15 h; relative humidity, 60 %).

We counted the number of seeds that had germinated every day. This allowed us to calculate germination success as the day to first germination and the proportion of germinated seeds. However, as the first seedling in each Petri dish emerged within 2 d after sowing, we did not analyse the day to first germination. On 22 August 2021, approx. 10 d after the first seedling had emerged and 5 d after the last seedling had emerged, we stopped the experiment. To assess treatment effects on seedling growth, we measured root length of the first germinated seedling within each Petri dish, 10 d after germination, using digital calipers (Harbin Measuring and Cutting Tools Group Co., Ltd).

Statistical analysis

All statistical analysis were done in R version 4.0.3 (R Core Team, 2020), unless stated otherwise.

Biomass production during pre-cultivation

To test whether species identity and competition affected biomass of the plants, we used a linear model. As explanatory model terms, we used species identity (*A. bidentata* and *C. sonchifolium*), competition treatment (no, intra- and interspecific competition) and their interaction. To test which competition treatments differed, we created two orthogonal contrasts. The first contrast tested the average effects of both forms of competition, and the second contrast compared intra- vs. interspecific competition. As residual plots showed that the two species differed in their variance, we used the varIdent function within the gls function of the nlme package to allow different variances for each species (Pinheiro *et al.*, 2020). In this way, we avoided violation of the assumption of homoscedasticity (Zuur *et al.*, 2009).

Metabolomic data

To test for differences in metabolomic profiles between species and among competition treatments, we performed a principal component analysis (PCA) on all compounds detected using the prcomp function in R. The data were unit variance scaled prior to analysis. As one of the samples from the interspecific competition treatment of *C. sonchifolium* clustered with *A. bidentata* (Supplementary data Fig. S1), which could indicate possible contamination of the sample or a mix up of samples, we also performed the PCA without this outlier. As the overall results were very similar (Supplementary data Table S2), we report on the PCA without the outlier in the text. To test whether the PC1 and PC2 scores differed between species and among competition treatments, we used linear models. To account for heterogeneity of variance, we allowed the variance to differ among the three competition treatments for PC1 and between the two species for PC2, using the varIdent function within the gls function of the nlme package (Zuur *et al.*, 2009; Pinheiro *et al.*, 2020).

In a next step, to test for differences in metabolomic profiles between species and among competition treatments, we used hierarchical cluster analysis on sub-sets of differently regulated metabolites. We did this for both species jointly and for each species separately. Metabolites were considered to be differently regulated between groups when their VIP was ≥1 and absolute $\log_2$-fold change was ≥1, as based on orthogonal partial least squares discriminant analysis (OPLS-DA) implemented in the MetaboAnalyst R package (version 1.0.1; Pang *et al.*, 2020). The data were $\log_2$-transformed and mean centred before OPLS-DA and, to avoid overfitting, a permutation test (200 permutations) was performed. The subsequent hierarchical cluster analyses on the differently regulated metabolites, and the visualization using heatmaps and dendrograms were done in the ComplexHeatmap package (version 2.8.0; Gu *et al.*, 2016).

Germination rate and seedling growth

The proportion of germinated seeds and root length of the first seedling were analysed with a binomial generalized linear mixed model (GLMM) and a linear mixed model (LMM), respectively. As fixed terms, we included 'test species' (*A. bindetata* and *C. sonchifolium*), 'extract type' (control, *Achyanthes* without competition, *Achyanthes* with intraspecific competition, *Achyanthes* with interspecific competition, *Crepidiastrum* without competition, *Crepidiastrum* with intraspecific competition and *Crepidiastrum* with interspecific competition) and their interaction. To account for non-independence of Petri dishes with aqueous extract made from the same plant individual (or the PEG control), we included 'individual' as a random factor. However, as the variance in germination explained by 'individual' was zero, it was removed from the final model.

To test more specifically which of the seven aqueous extract treatments differed, we created six orthogonal contrasts for 'extract type'. The first contrast tested whether the presence of an aqueous plant extract had an effect by comparing the PEG control with the average of all aqueous plant extracts. The second contrast tested whether there was a difference between the average of the three *Achyranthes* extracts and the average of the three *Crepidiastrum* extracts. The third contrast tested among the three *Achyranthes* extracts, whether there was a difference between the extract made from competition-free plants and the average of extracts made from plants grown with intra- and interspecific competition. The fourth contrast tested whether there was a difference between extracts made from plants grown with intra- and interspecific competition. The fifth and sixth contrasts were like the third and fourth but then for the *Crepidiastrum* extracts. To improve normality of the residuals in the analysis of root length, it was log-transformed. To account for heterogeneity of variance, we allowed the variance to differ between the two test species and among the extract types, using the varComb and varIdent functions within the lme function of the nlme package (Zuur *et al.*, 2009; Pinheiro *et al.*, 2020). The significances of the fixed factors and the contrasts were tested with log-likelihood ratio tests, comparing models with and without the term of interest (Zuur *et al.*, 2009).

When germination or root length of one of the two test species differed significantly among the extracts of plants that had experienced different competition treatments (i.e. when the third, fourth, fifth or sixth contrast described above was significant), we tested for each of the differently expressed compounds whether it correlated with germination or root length. This could only be done for the sub-set of Petri dishes for which we had metabolomics data of the extracts used. To account for multiple comparisons, we applied the Benjamini and Hochberg false discovery rate procedure, using $\alpha = 0.05$ (Benjamini and Hochberg, 1995).

RESULTS

Biomass in the competition experiment

For both *A. bidentata* and *C. sonchifolium*, the presence of a competitor significantly reduced individual biomass, but it did not matter whether the competitor was conspecific or heterospecific (Table 1; Fig. 1).

Metabolomics

Metabolomics analysis identified a total of 719 compounds in the aqueous extracts of the two species. The first two PC axes explained 45.4 % of the variation in metabolomes. Based on PC1, the metabolomic contents of the aqueous extracts differed between the two species (Fig. 2; log-likelihood ratio test of PC1 species effect: $\chi^2 = 112.51$, $P < 0.001$, Supplementary data Table S2b). There were no differences among the competition treatments for *C. sonchifolium* (Fig. 2). However, for *A. bidentata*, there was considerable variation along PC2, with higher values for the samples of plants grown with interspecific competition than for those grown with intraspecific competition (Fig. 2; log-likelihood ratio test of PC2 species × intra- vs. interspecific competition contrast: $\chi^2 = 10.08$, $P = 0.001$, Supplementary data Table S2b).

Hierarchical clustering on the metabolomics data of both species jointly also separated the two species, but did not reveal differences among the three competition treatments within each species (Supplementary data Fig. S2). However,

TABLE 1. *Results of a linear model testing the effect of species identity and competition treatments on individual biomass at the end of the competition experiment*

Fixed effects	d.f.	χ^2	*P*
Species	1	41.77	**<0.001**
Competition	2	6.92	**0.031**
Without vs, with competition	*1*	*5.99*	***0.014***
Intra- vs. interspecific competition	*1*	*1.07*	*0.301*
Species × Competition	2	2.10	0.350
Species × without vs. with competition	*1*	*2.09*	*0.149*
Species × intra- vs. interspecific competition	*1*	*0.02*	*0.902*

The orthogonal contrasts for competition treatment are shown in italics. *P*-values < 0.05 are shown in bold. To account for heterogeneity of variance, the variance was allowed to differ between the species. The s.d. of *C. sonchifolium* was 0.260 times the s.d. of *A. bidentata*

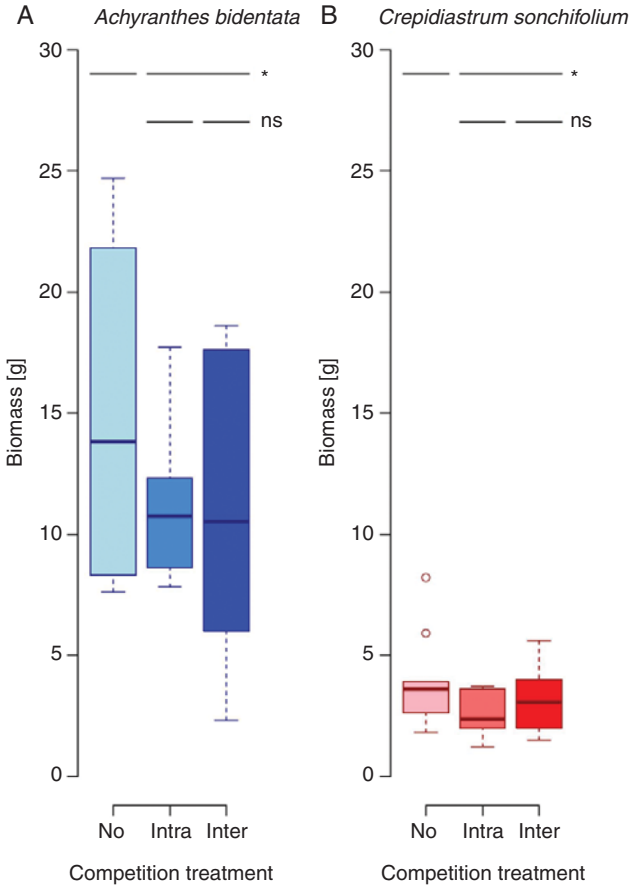

FIG 1. Boxplots showing the effects of the competition treatments on the total biomass of (A) *Achyranthes bidentata* and (B) *Crepidiastrum sonchifolium*. Boxes show the interquartile range around the median (thick horizontal line), whiskers extend to 1.5× the interquartile range and circles indicate outliers. The orthogonal contrasts of the competition treatments and their significances, for each species separately, are indicated with horizontal lines. *$P < 0.05$, n.s.: $P > 0.05$.

hierarchical clustering for each species separately revealed differences among the competition treatments. For *A. bidentata*, chemical composition of plants grown without competition and those grown with intraspecific competition did not differ, but the chemical composition of plants grown with interspecific

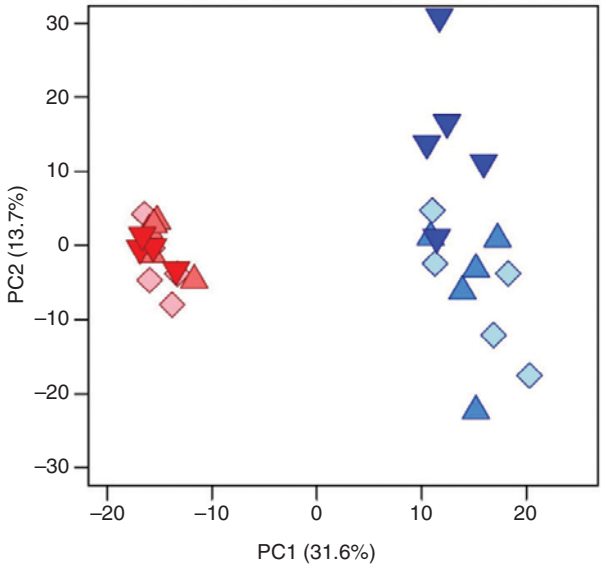

FIG. 2. Results of unsupervised principal component analysis on the metabolomics data, showing the scores along the first two PC axes. The samples of *Achyranthes bidentata* are shown in shades of blue, and the samples of *Crepidiastrum sonchifolium* are shown in shades of red. Samples of plants grown without competition are shown with diamond symbols with a light shade, samples of plants grown with intraspecific competition are shown with upright triangles with an intermediate shade and samples of plants grown with interspecific competition are shown with inverted triangles with a dark shade.

competition differed from those of the other two treatments (Supplementary data Fig. S3). For *C. sonchifolium*, the hierarchical clustering separated all three competition treatments, and the interspecific competition treatment differed the most from the other two treatments (Supplementary data Fig. S4).

Germination rate and seedling growth

More seeds germinated for *C. sonchifolium* than for *A. bidentata*, and the species responded differently to the extract types (Table 2; Fig. 3A, B). For both species, the proportion of germinated seeds on average did not differ between the PEG control and the aqueous extracts (Table 2; Fig. 3A, B). However, while germination of *C. sonchifolium* was lower on conspecific

TABLE 2. *Results of a GLM for proportion of germinated seeds, and an LMM for log(root length)*

		Germination		Log(root length)	
Fixed effects	d.f.	χ^2	***P***	χ^2	***P***
Test species	1	19.87	**<0.001**	5.08	**0.024**
Extract type	6	7.80	0.253	31.52	**<0.001**
Control vs. extract	*1*	*1.50*	*0.220*	*14.24*	***<0.001***
Achyranthes vs. Crepidiastrum	*1*	*2.06*	*0.151*	*7.67*	***0.006***
Achyrantes without vs. with comp.	*1*	*2.59*	*0.108*	*7.06*	*0.008*
Achyrantes intra- vs. interspecific comp.	*1*	*0.400*	*0.527*	*3.72*	*0.054*
Crepidiastrum without vs. with comp.	*1*	*0.992*	*0.319*	*5.13*	***0.023***
Crepidiastrum intra- vs. interspecific comp.	*1*	*0.274*	*0.601*	*0.41*	*0.524*
Test species × Extract type (T × E)	6	19.72	**0.003**	36.98	**<0.001**
T × Control vs. extract	*1*	*1.04*	*0.309*	*5.73*	*0.017*
T × *Achyranthes* vs. *Crepidiastrum*	*1*	*9.78*	***0.002***	*0.205*	*0.651*
T × *Achyrantes* without vs. with comp.	*1*	*8.35*	***0.004***	*18.27*	***<0.001***
T × *Achyrantes* intra- vs. interspecific comp.	*1*	*0.08*	*0.782*	*13.02*	***<0.001***
T × *Crepidiastrum* without vs. with comp.	*1*	*0.0004*	*0.984*	*0.95*	*0.330*
T × *Crepidiastrum* intra- vs. interspecific comp.	*1*	*1.02*	*0.313*	*0.08*	*0.774*
Random effects				SD	
Individual used for extract				0.2787	
Residual				0.3524*	

The orthogonal contrasts for extract type are shown in italics. *P*-values < 0.05 are shown in bold

*The residual s.d. was allowed to vary for the two test species and for the different extract types. The one shown is for test species *A. bidentata* and extract *Achyanthes* with interspecific competition. The multiplication factors for the s.d.s of the other factor levels are shown in Supplementary data Table S1.

than on heterospecific extracts, this was not the case for *A. bidentata* (Table 2; Fig. 3A, B). Nevertheless, *A. bidentata* had a reduced germination on conspecific extracts, when it had previously been grown with intra- or interspecific competition (Table 2; Fig. 3A, B). Of the 114 compounds differently expressed among the competition treatments of *A. bidentata*, six compounds were significantly correlated with germination of *A. bidentata*, but none remained significant after correction for multiple comparisons (Supplementary data Table S3).

Averaged over all treatments, 10-day-old seedlings of *A. bidentata* had slightly longer roots than those of *C. sonchifolium* (Table 2; Fig. 3C, D). Root length was significantly affected by the different extract types, and some of these effects differed between species (Table 2). For both species, root length was significantly reduced by the presence of plant extracts, relative to the PEG control (Table 2; Fig. 3C, D). For *A. bidentata*, the aqueous extracts had a stronger negative effect on root length when they were heterospecific instead of conspecific (Table 2; Fig. 3C). Moreover, root length of *A. bidentata* was smaller when exposed to conspecific extracts of plants that had experienced competition, particularly interspecific competition (Table 2; Fig. 3C). Of the 114 compounds differently expressed among the competition treatments of *A. bidentata*, 22 compounds were significantly correlated with root length of *A. bidentata* (six positively and 16 negatively), but none of these correlations remained significant after correction for multiple comparisons (Supplementary data Table S4). For *C. sonchifolium*, root length was more strongly reduced by conspecific extracts when the plants had previously experienced competition (Table 2; Fig. 3D). Of the 75 compounds differently expressed among the competition treatments of *C. sonchifolium*, 11 were significantly correlated with root length of *C. sonchifolium* (nine positively and two negatively), and three of the positive correlations remained significant after correction for multiple comparisons (Supplementary data Table S5). These were the compounds dihydroferulic acid, 3,4-dimethoxyphenyl acetic acid and (*R*)-(–)-2-phenylpropionic acid.

DISCUSSION

Metabolomics analysis of our aqueous plant extracts revealed differences in chemical profiles between the two study species, and showed that competition treatments modified these profiles (Fig. 2). For both species, we found negative allelopathic effects of aqueous plant extracts on germination and root length; the strenghts of these effects differed among the plant extract types. For root length of *A. bidentata*, heterospecific allelopathy was stronger than conspecific allelopathy (Fig. 3C), but the reverse was true for germination of *C. sonchifolium* (Fig. 3B), suggesting that extracts of *C. sonchifolium* had stronger negative allelopathic effects than those of *A. bidentata*. Heterospecific allelopathic effects were not dependent on the competition treatments. However, conspecific allelopathic effects on germination of *A. bidentata* (Fig. 3A) and root length of both species (Fig. 3C, D) were stronger when the aqueous extracts were made from plants grown with competition. For

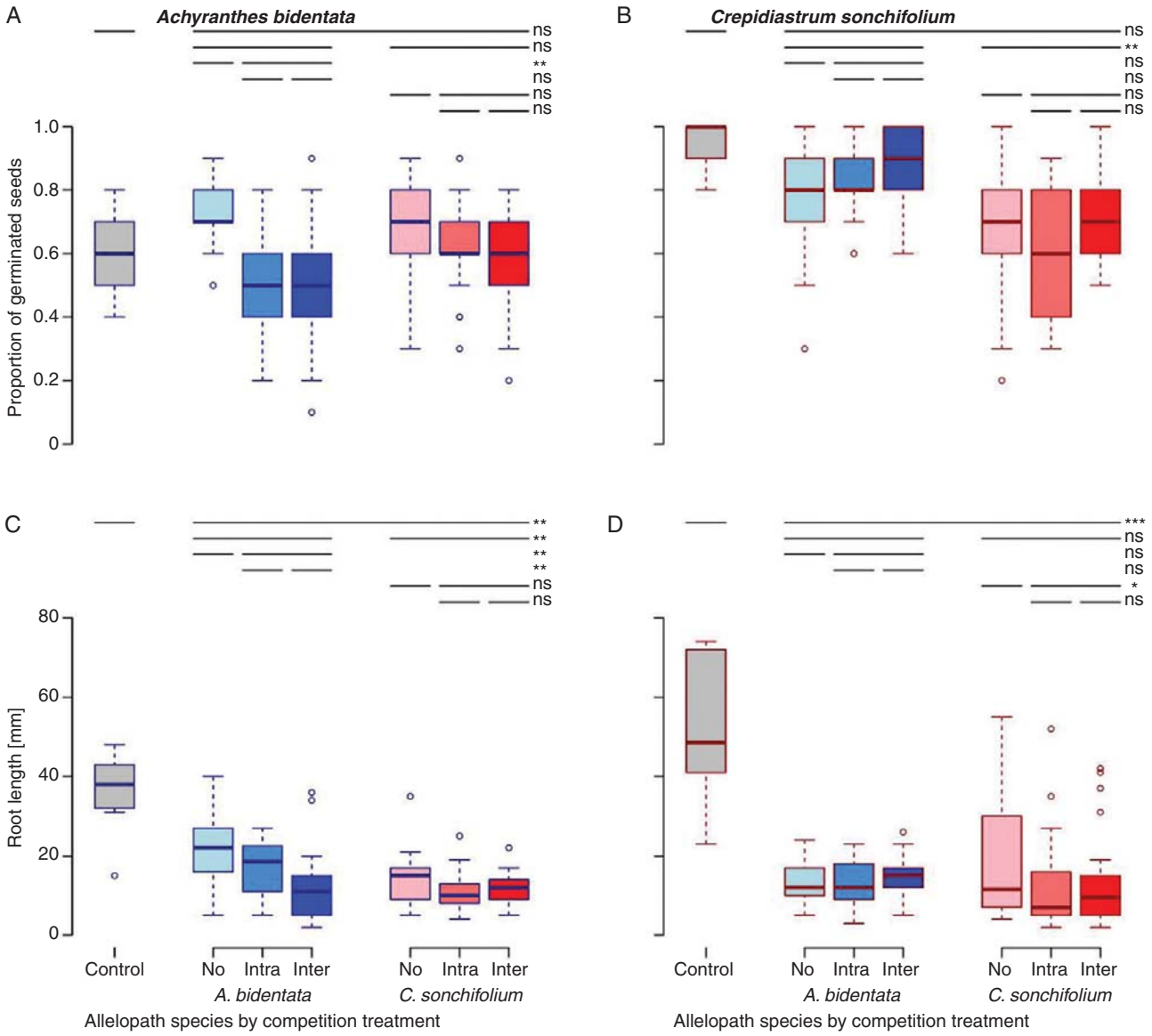

FIG. 3. Boxplots showing the effects of the aqueous extracts from the different allelopath species by competition treatments on the proportion of germinated seeds of (A) *Achyranthes bidentata* and (B) *Crepidiastrum sonchifolium*, and on root length of 10-day-old seedlings of (C) *A. bidentata* and (D) *C. sonchifolium*. Boxes show the interquartile range around the median (thick horizontal line), whiskers extend to 1.5× the interquartile range and circles indicate outliers. The orthogonal contrasts of the aqueous extract treatments and their significances, for each species separately, are indicated with horizontal lines. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s.: $P > 0.05$.

A. bidentata, this effect on root length was strongest when the plants had experienced interspecific instead of intraspecific competition. So, our study shows that competition can modify the chemical profiles of plants, and that this was correlated with stronger conspecific allelopathy.

Due to costs of producing defence compounds, there frequently is a trade-off between growth and defence (Kempel *et al.*, 2011; Zust and Agrawal, 2017). Similarly, one might expect that the production of allelochemicals is costly, and that their production therefore would only be induced when plants experience competition and allelopathy would be beneficial (Novoplansky, 2009; Kegge and Pierik, 2010). In our study, the presence of competitors not only reduced individual biomass (Fig. 1), but also changed the metabolome and was correlated with increased negative conspecific allelopathic effects on germination of *A. bidentata* and seedling growth of both species. Previous studies have shown that another biotic stress, herbivory, may result in increased allelopathy, possibly as a side effect of induced defences (Thelen *et al.*, 2005; Karban, 2007). Recently, Uesugi *et al.* (2019) found that competition with *Poa pratensis* induced the production of the allelochemical polyacetylene in *Solidago altissima*, but that this was only

the case for accessions of *S. altissima* from Australia, where the species is not native, and was stronger when the plants competed under low nutrient availability. Furthermore, several studies have shown that the production of allelochemicals increased when allelopathic crop plants grew in the presence of competing weeds (Dayan, 2006; Lu *et al.*, 2012; Gfeller *et al.*, 2018; Li *et al.*, 2020). For example, Li *et al* (2020) found that the production of allelochemicals by rice was induced in the presence of barnyard grass (*Echinochloa crus-galli*). However, none of these studies on induction of allelochemicals assessed whether the allelochemicals resulted in conspecific allelopathy (i.e. autoallelopathy).

Most studies have tested for heterospecific allelopathy and, although we also found evidence for this, conspecific allelopathy was stronger than heterospecific allelopathy for germination of *C. sonchifolium* (Fig. 3B). Moreover, conspecific allelopathy was induced by competition for germination of *A. bidentata* (Fig. 3A) and seedling growth of both species (Fig. 3C, D). Yuan *et al.* (2021) recently showed in a study on pairwise reciprocal allelopathy between five alien and five native species that negative allelopathic effects on germination were strongest for conspecifics. One potential mechanism for our finding that competition induced increased autoallellopathy is provided by the biochemical recognition hypothesis (Renne *et al.*, 2004, 2014). This hypothesis posits that metabolites produced by plants might be used by seeds to eavesdrop on which species are present, including conspecifics. If they detect that there is a strong competitor among the resident species, they may delay germination until better establishment conditions occur. In support of this hypothesis, it has been shown that seeds indeed can sense competitors (Preston and Baldwin, 1999; Renne *et al.*, 2014), facilitators (Yannelli *et al.*, 2020) and symbiotic hosts (Plakhine *et al.*, 2009) via phytochemicals released by conspecific or heterospecific neighbours. Souza-Alonso *et al.* (2017) even showed that volatile organic compounds released by the flowers of *Acacia dealbata* could reduce conspecific germination and seedling growth. The fact that we also found competition-induced conspecific allelopathic inhibition of seedling growth (Fig. 3C, D) suggests that metabolites in the environment are not only used to eavesdrop on neighbours to regulate germination but might also have phytotoxic effects. Moreover, critical to the idea that seeds delay their germination when they recognize the presence of competitors is that the seeds become dormant and thus remain viable (Cohen, 1967). As we did not test this, we recommend that future studies on induced allelopathy test the viability of seeds that did not germinate.

As conspecific plants have the same resource requirements, intraspecific competition is usually more intense than interspecific competition (Adler *et al.*, 2018). Intraspecific resource competition therefore results in negative density dependence of population growth (Keddy, 2001). Conspecific allelopathy (i.e. interference competition) that exceeds heterospecific allelopathy, as found in our study, could similarly result in negative density-dependent population growth. Moreover, at the same time, it could be a mechanism to avoid intraspecific resource competition.

Plants produce thousands of compounds, and not all of them may be exuded into the environment or remain stable. Therefore, it is challenging to study allelopathic interactions, and different approaches have been applied. As most studies on allelopathy have used aqueous plant extracts (Zhang *et al.* 2021), we also used such extracts to increase comparability among studies. This does not mean that the use of aqueous extracts is the most realistic approach. However, our main interest here was to test for competition-induced changes of the plant metabolome and how this could affect allelopathic interactions. Future studies should also test for competition-induced allelopathy using more realistic approaches such the collection and application of root exudates and leaf washes.

To gain insights into possible mechanisms underlying the observed competition-induced allelopathy, we analysed the metabolomes of the plant extracts. When we used all 719 compounds identified, PCA revealed a clear separation between the two species along PC axis 1 (Fig. 2). For the competition treatments within species, we only found that along PC axis 2, the metabolomes of *A. bidentata* plants that had experienced interspecific competition differed from those of the other *A. bidentata* plants (Fig. 2). The small differences among competition treatments might reflect that the PCA included all compounds, many of which were not differentially expressed. However, hierarchical clustering based on only those compounds that were differentially expressed among competition treatments within each species revealed a stronger separation of the competition treatments. For *A. bidentata* and *C. sonchifolium*, 114 and 77 compounds, respectively, were differently regulated among the competition treatments, and for both species the production was upregulated in response to competition for 73 % of the compounds (Supplementary data Figs S3 and S4). This suggests that plants invested more in secondary compound production when they experienced competition.

We found that some of the compounds differentially expressed among competition treatments correlated with allelopathic effects on germination and seedling growth. However, after correction for multiple comparisons, none of these correlations was significant for *A. bidentata*, and only three correlations [i.e. the positive correlations of root length with dihydroferulic acid, 3,4-dimethoxyphenyl acetic acid and (*R*)-(–)-2-phenylpropionic acid] remained significant for *C. sonchifolium*. For two of these compounds, we are not aware of any previous reports about potential allelopathic effects, but, for phenylpropionic acid, potential autoallelopathic effects have been previously reported for cucumber (*Cucumis sativus*; Yu *et al.*, 2003). However, in our study all three compounds had positive effects on root length, and were not upregulated but downregulated by competition. This could suggest that competition does not induce the production of compounds with negative allelopathic effects but inhibits the production of compounds with positive allelopathic effects on seedling growth. On the other hand, it could also be that none of the single compounds is responsible but that it is the cocktail of the differentially expressed compounds.

When allelopathy was induced by competition, in most cases it did not matter whether it was intra- or interspecific competition. This could suggest that the increased production of compounds that have negative allelopathic effects, or the reduced production of compounds that have postive allelopathic effects, is a general response to reduced resource availability. However, for seedling growth of *A. bidentata*, the aqueous extracts made

of conspecifics that had experienced interspecific competition with *C. sonchifolium* had stronger negative effects than those made of conspecifics that had experienced intraspecific competition (Fig. 3C). It has previously been shown that the identity of neighbouring plants can influence plant biochemistry involved in defence against herbivores (Metlen *et al.*, 2009; Callaway *et al.*, 2010). Our results thus suggest that this might also be the case for plant biochemistry involved in allelopathic interactions.

In conclusion, we showed that plants change their metabolomic profiles in response to competition, and that this correlated with allelopathic inhibition of conspecific seedling recruitment. We suggest that this autoallelopathy could function as a mechanism to avoid strong competition by keeping the seeds in a dormant state. However, whether this is the case will require more research.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: multiplication factors to calculate the residual standard deviations for each factor level of the LMM of log(rooth length). Table S2: results of LMs testing the effect of species identity and competition treatment on PC1 and PC2 of unsupervised PCAs on the metabolomic data. Table S3: metabolomic compounds that were differently expressed among *Archyranthes bidentata* plants that experienced different competition treatments and that were significantly correlated with germination rates of *A. bidentata*. Table S4: metabolomic compounds that were differently expressed among *Archyranthes bidentata* plants that experienced different competition treatments ($n = 114$; see Supplemetary data Fig. S3) and that were significantly correlated with root lengths of *A. bidentata* seedlings. Table S5: metabolomic compounds that were differently expressed among *Crepidiastrum sonchifolium* plants that experienced different competition treatments and that were significantly correlated with root lengths of *C. sonchifolium* seedlings. Appendix 1: metabolomic analysis of the plant extracts. Figure S1: results of unsupervised principal component analysis on the metabolomic data, showing the scores along the first two PC axes. Figure S2: metabolomic differences for species *Achyranthes bidentata* and *Crepidiastrum sonchifolium* under various competition treatments. Figure S3: metabolomic differences for species *Achyranthes bidentata* under various competition treatment. Figure S4: metabolomic differences for species *Crepidiastrum sonchifolium* under various competition treatments.

ACKNOWLEDGEMENTS

The authors declare no conflicts of interests. Author contributions: M.v.K. and J.L. came up with the idea. M.v.K. and Y.L. designed the experiment. Y.L. performed the experiment. M.v.K. and Y.L. analysed the data. Y.L. and M.v.K. wrote the paper with contributions from J.L. All authors gave final approval for publication. Data availability statement: the data will be made available through Figshare.

FUNDING

This work was supported by the Research Foundation for Advanced Talents in Taizhou University (grant no. 0104010008).

LITERATURE CITED

Adler PB, **Smull D**, **Beard KH**, ***et al***. **2018**. Competition and coexistence in plant communities: intraspecific competition is stronger than interspecific competition. *Ecology Letters* **21**: 1319–1329. doi:10.1111/ele.13098

Aschehoug ET, **Brooker R**, **Atwater DZ**, **Maron JL**, **Callaway RM. 2016**. The mechanisms and consequences of interspecific competition among plants. *Annual Review of Ecology Evolution & Systematics* **47**: 263–281.

Benjamini Y, **Hochberg Y. 1995**. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B: Methodological* **57**: 289–300.

Callaway RM, **Aschehoug ET. 2000**. Invasive plants versus their new and old neighbors: a mechanism for exotic invasion. *Science* **290**: 521–523. doi:10.1126/science.290.5491.521

Callaway RM, **Ridenour WM. 2004**. Novel Weapons: invasive success and the evolution of increased competitive ability. *Frontiers in Ecology and the Environment* **2**: 436–443. doi:10.1890/1540-9295(2004)002[0436:nwisat]2.0.co;2

Callaway RM, **Erick G**, **Lewis MR**, ***et al***. **2010**. Plant neighbor identity influences plant biochemistry and physiology related to defense. *BMC Plant Biology* **10**: 115.

Cohen D. 1967. Optimizing reproduction in a randomly varying environment when a correlation may exist between the conditions at the time a choice has to be made and the subsequent outcome. *Journal of Theoretical Biology* **16**: 1–14. doi:10.1016/0022-5193(67)90050-1

Dayan FE. 2006. Factors modulating the levels of the allelochemical sorgoleone in *Sorghum bicolor*. *Planta* **224**: 339–346. doi:10.1007/s00425-005-0217-5

Duke SO. 2010. Allelopathy: current status of research and future of the discipline: a commentary. *Allelopathy Journal* **25**: 17–30.

Gause GF. 1934. *The struggle for existence*. Baltimore: Williams & Wilkins.

Gfeller A, **Glauser G**, **Etter C**, **Signarbieux C**, **Wirth J. 2018**. *Fagopyrum esculentum* alters its root exudation after *Amaranthus retroflexus* recognition and suppresses weed growth. *Frontiers in Plant Science* **9**: 50. doi:10.3389/fpls.2018.00050

Gu Z, **Eils R**, **Schlesner M. 2016**. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**: 2847–2849. doi:10.1093/bioinformatics/btw313

Hierro JL, **Callaway RM. 2021**. The ecology importance of allelopathy. *Annual Review of Ecology, Evolution and Systematics* **52**: 25–45.

Hutchinson GE. 1959. Homage to Santa Rosalia or why are there so many kinds of animals? *American Naturalist* **93**: 145–159.

Inderjit, **Nilsen ET. 2003**. Bioassays and field studies for allelopathy in terrestrial plants: progress and problems. *Critical Review in Plant Science* **22**: 221–238.

Inderjit, **Wardle DA**, **Karban R**, **Callaway RM. 2011**. The ecosystem and evolutionary contexts of allelopathy. *Trends in Ecology & Evolution* **26**: 655–662.

Inderjit, **Simberloff D**, **Kaur H**, **Kalisz S**, **Bezemer TM. 2021**. Novel chemicals engender myriad invasion mechanisms. *New Phytologist* **232**: 1184–1200.

Karban R. 2007. Experimental clipping of sagebrush inhibits seed germination of neighbours. *Ecology Letters* **10**: 791–797. doi:10.1111/j.1461-0248.2007.01068.x

Kato-Noguchi H. 2011. Barnyard grass-induced rice allelopathy and momilactone B. *Journal of Plant Physiology* **168**: 1016–1020. doi:10.1016/j.jplph.2010.12.021

Keddy PA. 2001. *Competition: population and community biology series*, 2nd edn. Dordrecht: Springer.

Kegge W, **Pierik R. 2010**. Biogenic volatile organic compounds and plant competition. *Trends in Plant Science* **15**: 126–132. doi:10.1016/j.tplants.2009.11.007

Kempel A, **Schädler M**, **Chrobock T**, **Fischer M**, **van Kleunen M. 2011**. Trade-offs associated with constitutive and induced plant resistance against herbivory. *Proceedings of the National Academy of Sciences, USA* **108**: 5685–5689.

Kong C, **Li H**, **Hu F**, **Xu X**, **Wang P. 2006**. Allelochemicals released by rice roots and residues in soil. *Plant and Soil* **288**: 47–56.

Lankau RA, **Kliebenstein DJ. 2009**. Competition, herbivory and genetics interact to determine the accumulation and fitness consequences of a defence metabolite. *Journal of Ecology* **97**: 78–88.

Li L, **Zhao H**, **Kong C. 2020**. (–)-Loliolide, the most ubiquitous lactone, is involved in barnyardgrass-induced rice allelopathy. *Journal of Experimental Botany* **71**: 1540–1550. doi:10.1093/jxb/erz497

Lu C, **Liu X**, **Xu J**, ***et al***. **2012**. Enhanced exudation of DIMBOA by wheat seedlings alone and in proximity to wild oat (*Avena fatua*) and flixweed (*Descurainia sophia*). *Weed Science* **60**: 360–365.

Metlen KL, **Aschehoug ET**, **Callaway RM. 2009**. Plant behavioural ecology: dynamic plasticity in secondary metabolites. *Plant, Cell & Environment* **32**: 641–653. doi:10.1111/j.1365-3040.2008.01910.x

Novoplansky A. 2009. Picking battles wisely: plant behaviour under competition. *Plant, Cell & Environment* **32**: 726–741.

Oduor A, **van Kleunen M**, **Stift M. 2020**. Allelopathic effects of *Brassica nigra* in both its native and invasive ranges do not support the novel-weapons hypothesis. *American Journal of Botany* **107**: 1106–1113.

Pang Z, **Chong J**, **Li S**, **Xia J. 2020**. MetaboAnalystR 3.0: toward an optimized workflow for global metabolomics. *Metabolites* **10**: 186. doi:10.3390/metabo10050186

Pinheiro J, **Bates D**, **DebRoy S**, **Sarkar D**, **R Core Team**. **2020**. *nlme: linear and nonlinear mixed effects models. R package version 3.1-149*. https://CRAN.R-project.org/package=nlme

Plakhine D, **Ziadna H**, **Joel DM. 2009**. Is seed conditioning essential for Orobanche germination? *Pest Management Science* **65**: 492–496. doi:10.1002/ps.1716

Preston CA, **Baldwin IT. 1999**. Positive and negative signals regulate germination in the post-fire annual *Nicotiana attenuata*. *Ecology* **80**: 481–494. doi:10.2307/176627

Rabotnov TA. 1982. Importance of the evolutionary approach to the study of allelopathy. *Ekologia* **3**: 5–8 (Translated from Russian).

R Core Team. **2020**. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/

Renne IJ, **Rios BG**, **Fehmi JS**, **Tracy BF. 2004**. Low allelopathic potential of an invasive forage grass on native grassland plants: a cause for encouragement? *Basic and Applied Ecology* **5**: 261–269. doi:10.1016/j.baae.2003.11.001

Renne IJ, **Sinn BT**, **Shook GW**, ***et al***. **2014**. Eavesdropping in plants: delayed germination via biochemical recognition. *Journal of Ecology* **102**: 86–94.

Roberts KJ, **Anderson RC. 2001**. Effect of garlic mustard *Alliaria petiolata* (Beib. Cavara and Grande) extracts on plants and arbuscular mycorrhizal (AM) fungi. *The American Midland Naturalist* **146**: 146–152. doi:10.1674/0003-0031(2001)146[0146:eogmap]2.0.co;2

Song B, **Xiong J**, **Fang C**, ***et al***. **2008**. Allelopathic enhancement and differential gene expression in rice under low nitrogen treatment. *Journal of Chemical Ecology* **34**: 688–695. doi:10.1007/s10886-008-9455-x

Souza-Alonso P, **Rodríguez J**, **González L**, **Lorenzo P. 2017**. Here to stay. Recent advances and perspectives about Acacia invasion in mediterranean areas. *Annals of Forest Science* **74**: 55.

Thelen GC, **Vivanco JM**, **Newingham B**, ***et al***. **2005**. Insect herbivory stimulates allelopathic exudation by an invasive plant and the suppression of natives. *Ecology Letters* **8**: 209–217. doi:10.1111/j.1461-0248.2004.00713.x

Tilman D, **Isbell F**, **Cowles JM. 2014**. Biodiversity and ecosystem functioning. *Annual Review of Ecology Evolution and Systematics* **45**: 471–493.

Uesugi A, **Johnson R**, **Kessler A. 2019**. Context-dependent induction of allelopathy in plants under competition. *Oikos* **128**: 1492–1502. doi:10.1111/oik.06389

Yannelli FA, **Novoa A**, **Lorenzo P**, **Roux J**, **Rodríguez J. 2020**. No evidence for novel weapons: biochemical recognition modulates early ontogenetic processes in native species and invasive Acacias. *Biological Invasions* **22**: 549–562.

Yu J, **Ye S**, **Zhang M**, **Hu W. 2003**. Effects of root exudates and aqueous root extracts of cucumber (*Cucumis sativus*) and allelochemicals, on photosynthesis and antioxidant enzymes in cucumber. *Biochemical Systematics and Ecology* **31**: 129–139.

Yuan L, **Li J**, **Yu F**, **Oduor A**, **van Kleunen M. 2021**. Allelopathic and competitive interactions between native and alien plants. *Biological Invasions* **23**: 3077–3090.

Zhang S, **Ding B. 1993**. *Zhejiang flora*. Hangzhou, China: Zhejiang Science and Technology Publishing House.

Zhang Z, **Liu Y**, **Yuan L**, **Weber E**, **van Kleunen M. 2021**. Effect of allelopathy on plant performance: a meta-analysis. *Ecology Letters* **24**: 348–362. doi:10.1111/ele.13627

Züst T, **Agrawal AA. 2017**. Trade-offs between plant growth and defense against insect herbivory: an emerging mechanistic synthesis. *Annual Review of Plant Biology* **68**: 513–534. doi:10.1146/annurev-arplant-042916-040856

Zuur A, **Ieno EN**, **Walker N**, **Saveliev AA**, **Smith GM. 2009**. *Mixed effects models and extensions in ecology with R*. Berlin: Springer.