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Biogeographic differences in the allelopathy of leaf surface extracts of an invasive weed

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Abstract

Allelopathy, the release of chemicals by plants that inhibit the germination and growth of competing species, can be an important trait for invasive success. However, little is known about potential biogeographical differences in allelopathy due to divergent regional eco-evolutionary histories. To test this, we examined the allelochemical potential of the highly invasive species *Centaurea solstitialis* from six world regions including native (Spain, Turkey) and non-native ranges (Argentina, Chile, California and Australia). Seeds from several populations in each region were collected and grown under common garden conditions. Allelopathic potential and chemical composition of three leaf extract concentrations of *C. solstitialis* from each region: 0.25%, 0.5% 0.75% (w/v-1) were assessed on the phytometer *Lactuca sativa*. The main allelochemicals present in the leaf-surface extract were sesquiterpene lactones that varied in major constitutive compounds across regions. These leaf extracts had strong inhibitory effects on *L. sativa* seed germination and net growth. Summed across regions, the 0.25% concentration suppressed germination by 72% and radicle elongation by 66%, relative to the controls. At the 0.5% concentration, no seeds germinated when exposed to extracts from the non-native ranges of Argentina and Chile, whereas germination and radicle growth were reduced by 98% and 89%, respectively, in the remaining regions, relative to controls. Germination and seedling growth were completely inhibited at the 0.75% concentration extract for all regions. Some non-native regions were characterized by relatively lower concentrations of allelochemicals, suggesting that there is biogeographical variation in allelopathic expression. These findings imply that rapid selection on the biochemical signatures of an exotic invasive plant species can be highly region-specific across the world.

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ABSTRACT

Allelopathy, the release of chemicals by plants that inhibit the germination and growth of competing species, can be an important trait for invasive success. However, little is known about potential biogeographical differences in allelopathy due to divergent regional eco-evolutionary histories. To test this, we examined the allelochemical potential of the highly invasive species *Centaurea solstitialis* from six world regions including native (Spain, Turkey) and non-native ranges (Argentina, Chile, California and Australia). Seeds from several populations in each region were collected and grown under common garden conditions. Allelopathic potential and chemical composition of three leaf extract concentrations of *C. solstitialis* from each region: 0.25%, 0.5% 0.75% (w/v⁻¹) were assessed on the phytometer *Lactuca sativa*. The main allelochemicals present in the leaf-surface extract were sesquiterpene lactones that varied in major constitutive compounds across regions. These leaf extracts had strong inhibitory effects on *L. sativa* seed germination and net growth. Summed across regions, the 0.25% concentration suppressed germination by 72% and radicle elongation by 66%, relative to the controls. At the 0.5% concentration, no seeds germinated when exposed to extracts from the non-native ranges of Argentina and Chile, whereas germination and radicle growth were reduced by 98% and 89%, respectively, in the remaining regions, relative to controls. Germination and seedling growth were completely inhibited at the 0.75% concentration extract for all regions. Some non-native regions were characterized by relatively lower concentrations of allelochemicals, suggesting that there is biogeographical variation in allelopathic expression. These findings imply that rapid selection on the biochemical signatures of an exotic invasive plant species can be highly region-specific across the world.

KEYWORDS: biogeographical contrasts, biotic resistance, leaf-surface chemicals, phytotoxicity, sesquiterpene lactones, yellow star-thistle

Introduction

The release of compounds in leaf leachates, root exudates, volatiles, and from decaying plant material (Weir et al. 2004) can contribute to competitive interactions and plant defense (Müller-Schärer et al. 2004; Aschehoug et al. 2016). Allelopathic compounds can defend against pathogens (Meepagala et al. 2006; Zhang et al. 2013), inhibit germination (Chon and Nelson 2010), suppress other plant species (Ridenour and Callaway 2001; Lankau 2012), deter herbivores (Thelen et al. 2005), enhance nutrient acquisition (Tharayil et al. 2009), and ameliorate abiotic stressors such as high light intensity (Izhaki 2002). Additionally, some of these compounds, like emodine, appear to have multiple functions at a time, such as insect anti-feeding agent, seed dispersal facilitator and plant growth inhibitor (Hasan 1988; Inoue et al. 1992; Izhaki 2002).

Allelopathy has been linked to successful exotic invasion by non-native species (Callaway and Ridenour 2004). For example, the root extracts of the invasive *Centaurea diffusa* suppress *A. thaliana* seedlings (Quintana et al. 2009), whereas its shoot extracts inhibit the germination and growth of several *Lolium* species (Muir and Majak 1983). In some cases, phytotoxic compounds, such as polyacetylenes have been isolated from the roots of exotic invaders (*Acroptilon repens*, Quintana et al. 2008) and from soils occupied by exotics (Dayan et al. 2010). Collectively, this evidence suggests that plant compounds are powerful agents that can influence the outcome of interactions with other plant competitors and with consumers, and play an important role in at least some invasion processes such as interference or competitive interactions with other resident species.

Invasive species can rapidly develop different sets of adaptations to the different non-native regions they colonize (Maron et al. 2004; Callaway and Ridenour, 2004; Graebner et al. 2012). If trait adaptation to local conditions is common, allelopathy should also experience selective forces both directly and indirectly via trade-offs and, in fact, the few studies that have compared allelopathy in native and non-native ranges have found significant differences (Yuan et al. 2012; Gruntman et al. 2015). However, previous studies tended to include one or two regional sites within the native or introduced ranges and found either increased allelopathy in the non-native range, or no differences (Gruntman et al. 2015). There is no reason to expect that all non-native regions will present similar levels of allelopathic effects, since diverging selective forces and trade-offs could result in biogeographical changes in the relative importance of allelopathy in each range and region. Additionally, there may be variation in metabolic profiles and allelochemical production due to biogeographic history (Callaway and Aschehoug 2000; Schemske 2010; Rosche et al. 2018) or variation in soil nutrients, drought or thermal stress (Sampaio et al. 2016), potentially resulting in different trait-shifts specific to ecologically unique regions. Therefore, it is crucial to conduct biogeographical experiments that compare allelochemical production and allelochemical effects for both native and introduced populations of invasive species.

Centaurea solstitialis (yellow starthistle, Asteraceae) is an ideal model species to study local adaptation rates and subsequent trait divergences. It is a Eurasian winter annual forb introduced throughout the world and highly invasive in the Americas. During its colonization history, the species had evolved many trait differences in its introduced areas, relative to the native range, such as increased seed size, germination timing and adult plant size (Graebner et

al. 2012, Hierro et al. 2013; Barker et al. 2017), and increased competitive ability (Montesinos and Callaway 2017), among others. Other species in the genus were found to be allelopathic (Callaway and Aschehoug 2000; Ni et al. 2010; Chen et al. 2013; Aschehoug et al. 2014), but previous experiments did not find evidence of root-mediated allelopathy in *C. solstitialis* (Carpenter 2007; Qin et al. 2007); however, some studies suggest that leaf leachates may be allelopathic (Zamora 1984; Gómez-González et al. 2009; Filipe et al. 2016). All aerial parts of *C. solstitialis* possess several sesquiterpene lactones (Stevens and Merrill 1985; Alvarez 2008; Sotes et al. 2015) which have well known pharmacological and biological inhibitory activity (Cheng et al. 1992; Özçelik et al. 2009), and that can act as an inducible chemical defense against herbivory (Beck et al. 2008; Smith and Beck 2013; Oster et al. 2015) or mediate competitive interactions with neighboring plants. In the Asteraceae, sesquiterpenes are usually secreted and stored in glandular trichomes of the leaves or in epidermic folds or cavities due to their high toxicity (Gofpert et al. 2005) but they may also occur in secretory ducts inside the leaf mesophyll (Bartoli et al. 2011). Nevertheless, this aspect remains poorly studied in *C. solstitialis*. Stevens and Merrill (1985) tested the individual effects of several sesquiterpene lactones isolated from fresh leaves of *C. solstitialis* from California on lettuce seeds germination and seedling growth and found them to possess growth regulatory activity, with low concentrations having stimulatory effects and higher concentrations displaying inhibitory effects. Hence, the chemicals present on the leaf surfaces of *C. solstitialis*, especially terpenoids, are potentially biologically relevant. They may present allelopathic activity, particularly as some of them have hydrophilic properties and can accumulate in the soil through rain or litter decomposition (Sotes et al. 2015). However, it is unknown whether *C. solstitialis*

leaf surface chemicals are involved in allelopathic inhibition of other plants, or if there is any biogeographical variation in the composition and activity of leaf surface chemicals.

We addressed knowledge gaps relative to the biogeography of the composition and allelopathic activity of invasive species by assessing (1) *C. solstitialis* leaf-surface chemistry for plants from six different native and non-native regions across the world, to determine whether there are biogeographical differences in allelochemical production, and (2) effects of leaf-extracts from these plants on seed germination and root growth of *Lactuca sativa*, a “phytometer” commonly used in allelopathic experiments, to test whether *C. solstitialis* leaf chemicals are effectively allelopathic.

Materials and methods

Plant material

Mature seeds of *Centaurea solstitialis* were collected in the field from several different maternal individuals from five natural populations in each of six different world regions, in the species’ native range (Turkey and Spain) and non-native ranges (California, Argentina, Chile and Australia) (Fig. 1 ; Table 1). Turkey is regarded as the geographic center of the species origin (Uygur et al. 2004), and Spain the primary source of seeds that colonized South America (Eriksen et al. 2014, Barker et al. 2017), while the remaining four regions represent important introductions, where the species is considered an invasive weed. Populations in each region were separated by at least 30 km within each region. Thus, our sampling was broad and considered the invasion pathways and historical relationships among different geographic regions.

Seeds from ten individuals from each of the 30 populations selected were germinated in a glasshouse at the Botanical Garden of the University of Coimbra, Portugal, in 50 cell plug trays containing standard potting soil (Substratos Profissionais, Leal & Soares S.A., Portugal). After 3 weeks, plants were transplanted into larger 2 L square plastic pots (1 individual per pot), filled with standard potting soil and kept in the glasshouse on top of flow benches for the remainder of the experiment. Plants experienced natural sunlight and Mediterranean warm climate in Coimbra and were watered three times per week to guarantee no water shortage. Chemical fertilizer was supplied once during the experiment (Fertiberia Jardin, Spain, NPK: 8-4-6 plus microelements) by applying 25 ml of solution to each pot. We noticed no sign of nutrient deficiency, drought, or herbivory, that could have potentially altered the production of chemical compounds in the leaves. Commercial seeds of *Lactuca sativa* (lettuce; Bionda Degli Ortolani, Vilmorin, France) were used as model species for testing the effects of leaf extracts on seed germination and radicle growth. This species is recommended for the assessment of ecological effects of toxic compounds by several agencies and protocols, including the US Environmental Protection Agency, the OECD, and ISO (US EPA 1996; ISO 1995; OECD 2003).

Leaf-surface extracts

Three months after germination of *C. solstitialis*, the five largest fresh and healthy-looking rosette leaves (typically 120-150 mm long by 35-40 mm wide), were harvested from each of ten individuals per population, from 5 populations in each region. Leaves from all ten individuals within each population were pooled and their surface chemicals solvent-extracted by soaking leaves in 500 mL of CH₂Cl₂ (dichloromethane) at room temperature for 30 seconds, enough time

to extract surface chemical compounds, but not enough to destroy tissues that would release internal leaf components (Sotes et al. 2015). Consequently, our leaf-surface extracts targeted those chemicals that are more likely to interact with other organisms. In contrast, crushed leaf extracts tend to include other constituent chemical compounds which could be less important for allelopathic interactions (Inderjit and Dakshini 1995). Extracts from each of the 30 world populations were then filtered through 598 Whatman filter paper to remove debris. A final leaf-surface extract per region, n=1 pooled sample for each of the six regions, was obtained by mixing together the extracts of the five populations within that region and concentrated using a vacuum rotary evaporator (IKA RV 8, Wilmington, USA). Plants and populations were pooled in order to obtain enough homogeneous extract from each region to conduct bioassays. Note that replication at the population level would have been desirable, because this would provide the opportunity to account for within-region variation (e.g., among and within populations). However, despite a design that accounted for within-region variation, the quantity of leaves obtained from individuals did not provide sufficient extract concentrations to accurately test for this, therefore we pooled extracts from populations within each region in order to develop the phytotoxicity bioassays. Between 2-4 mg of each of the six pooled extracts were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) on an Agilent Technologies 7820A System gas chromatograph linked to an Agilent Technologies 5975 Series MSD mass spectrometric detector as described by Sotes et al. (2015). The dried extract was stored in sterile glass vials at 4°C and used within one week in bioassays. Three different concentrations: 0.25%, 0.5% and 0.75% were obtained by re-suspending the dried extract in dichloromethane.

***Lactuca sativa* seed germination and radicle growth**

Our experiment involved 18 treatments (6 regions × 3 different concentrations) and two controls (positive and negative controls). Each treatment had three replicates and each control had five replicates. Although extracts from each population were used only for three replicates each, the number of populations (N=30), and the number of treatments resulted in a total number of experimental units of 64 (18 × 3 + 2 × 5). Thirty-six *L. sativa* seeds were placed on a Petri dish lined with one 90 mm Whatman filter paper (GE Healthcare) and moistened with 4 mL of plant extract, re-suspended in dichloromethane. We used distilled water as a negative control and dichloromethane as a positive control, with 4 mL of each applied to the filter paper, in order to control for potential effects of the extracting solvent in the germination tests. For all treatments, but the negative control, the organic solvent was evaporated in the fume hood followed by re-moistening of the filter paper with 3.5 mL of distilled water, following the standard protocol for germination bioassays (Vrchotová et al. 2011). Plates were sealed with parafilm to keep moisture in and stored at room temperature (25°C) in a completely randomized design. Germination was recorded daily for 14 days. A seed was categorized as germinated when a radicle of ≥ 1 mm was present. At the end of the experiment, we measured the length of the radicle of the germinated seeds to the nearest mm.

Data analysis

To quantify variation in chemical compounds among regions, we did a principal component analysis (standardized and centered PCA) on the chemical compounds that we found in our GC-MS analyses using the *vegan* 2.5-1 package (Oksanen et al. 2018) in R 3.5.1 (R Core

Team 2018), employing the `prcomp` function to compute principal component scores. We then plotted our PCA with the `ggbiplot` 0.55 package (Vu 2011) and colored the regions in the PCA plot according to their assignment to the native and non-native ranges. Further, we used the `psych` and `stats` packages in R (Revelle 2018) together with the `panel.cor`, `cor.test` and `panel.hist` functions to test for collinearity in the chemical dataset, generate pairwise scatter plots of all the variables and compute the Pearson correlation. Variables exhibiting significantly high correlation coefficients ($p\text{-value} \leq 0.01$) were dropped from the model and a PCA was performed on the remaining variables to assess the levels of variance explained by the two axes and the phytochemical profiles of each region.

We calculated two germination indices: percent germination and days to germination using spreadsheets and commands developed by Ranal et al. (2009). Data were checked for heteroscedasticity and normality by Levene's and Shapiro-Wilk tests (Levene 1960; Shapiro and Wilk, 1965). Data on seed germination (binomial) were analyzed with generalized linear models (GzLM), whereas data on untransformed radicle length (Gaussian) and days to germination (Poisson) were analyzed with linear models. To test for differences in the three response variables among regions, we ran models for each concentration with region as the fixed factor. If the models indicated significant differences among regions, we applied Tukey HSD post-hoc tests with $p\text{-values} < 0.05$ to infer which regions differed. To test for differences in the response variables between native vs. non-native ranges, we generated mixed-effects models (one for each concentration) with range as fixed factor and region as a random factor.

Results

Chemical compounds in leaf-surface extracts

GC-MS phytochemical analysis of *C. solstitialis* leaf-surface extracts identified three major classes of chemical compounds: alkanes, sesquiterpene lactones and pentacyclic triterpenoids (Table 2). The biologically important sesquiterpene lactones were the main class of substances. In total, we detected 24 compounds, including thirteen sesquiterpene lactones, three pentacyclic triterpenoids, and eight alkanes. The total unidentified fraction of the extracts ranged between 3.0-11% and the mass spectra of these extracts were similar to that of lactones.

The sesquiterpene lactone fraction represented more than 64% of the final extract for all regions, except California where it was 56%. *Centaurea solstitialis* from California exhibited the largest diversity of sesquiterpene lactones of all regions (12 out of a total of 13 sesquiterpene lactones detected; Table 3). In contrast, 11 sesquiterpene lactones were identified in the Spanish extract, nine in Turkey, Argentina and Chile, and eight in Australia (Table 3). *Repin* was the major compound of the sesquiterpene lactone fraction in extracts from plants from Argentina (32%) and Spain (26%) followed by the *repin*'s isomer *subluteolide*, in extracts from Australia (31%) and Turkey (27%). Conversely, *epoxyrepdiolide* (19%), *janerin* (19%), *subluteolide* (15%) and *repin* (12%) were the major compounds in the Chilean extract, respectively *repin* (13%) and *epoxyrepdiolide* (12%) of the Californian extract. Likewise, *solstitialin A-13 acetate* concentrations displayed higher values in extracts of native plants from Turkey and Spain (8% and 10%) than in non-native plants from the remaining four regions (1 - 2%), whereas *solstitialin A-3 acetate* was found only in the Turkish and Spanish extracts, but in much smaller amounts (under 1%). *Cynaropicrin* derivatives were found exclusively in leaf extracts from non-native

251 regions. Overall, *desacylcynaropicrin*, *aguerin B*, *cebellin F*, *cynaropicrin 3,4'-diacetate* and
 252 *cynaropicrin 4'-acetate* were present in very small amounts (under 1% of the final extract
 253 composition) across all regions (Table 3). To our knowledge, this is the first evidence of *cebellin*
 254 *F* presence in *C. solstitialis*, although *cebellin C* had been previously reported (Bruno et al. 2013).
 255 The concentrations of pentacyclic triterpenoids (waxes) were similar among regions (Table 4).
 256 Californian plants had high concentrations of alkanes (29%), whereas Chilean plants had lower
 257 concentrations (10%) comprising just three alkanes (all linear) out of a total of eight identified in
 258 this study (Tables 5). *Pentacosan* ($C_{25}H_{52}$) was identified only in the Californian extract.
 259 *Dotriacontane* ($C_{32}H_{66}$) and *tritriacontane* ($C_{33}H_{68}$) are reported for the first time in *C. solstitialis*
 260 (Table 5). The first and second axes of the PCA explained 66% of the inertia variance (Fig. 2). The
 261 first axis was mainly correlated with the compounds *janerin*, *epoxyrepdiolide*, and *cebellin F*, and
 262 therefore represents gradients in sesquiterpene lactones. The second axis was mainly correlated
 263 with *desacylcynaropicrin*. The PCA revealed a clustering of the two native regions in the lower
 264 right quarter, corresponding to high contents of *solstitialin-A3-acetate*, *solstitialin-A13-acetate*,
 265 and *hentriacontane* ($C_{31}H_{64}$). In contrast, the non-native regions were distributed widely across
 266 the other three quarters of the PCA plot. The collinearity test showed a significant positive
 267 relationship between *solstitialin-A3-acetate* and *solstitialin-A13-acetate* (correlation coefficient
 268 of 0.939), *acroptilin* and *aguerin B* (correlation coefficient of 0.922), *cynaropycrin-3-acetate* and
 269 $C_{29}H_{60.1}$ (correlation coefficient of 0.963) and *cynaropicrin-4-acetate* and *pentacosan* ($C_{25}H_{52}$)
 270 (correlation coefficient of 1) (Appendix S1 Figure S1). After dropping out *solstitialin-A3-acetate*,
 271 *aguerin B*, $C_{29}H_{60.1}$ and *cynaropicrin-4-acetate* from the model, the PCA explained 66% variance

in the two axes, similar to the initial PCA on all the variables, but revealed slightly different region clustering based on chemical compounds (Appendix S1 Figure 2).

Effects of leaf-extracts on *Lactuca sativa* germination

Lactuca sativa control seeds germinated at an overall rate of $99\% \pm 0.2\%$ (mean $\pm$ SE), thus neither deionized water nor dichloromethane affected germination. Overall, the average germination rate for *L. sativa* seeds exposed to the lowest leaf-extract concentration (0.25%) was 28%, followed by an overall germination rate of 2.0% for the 0.5% extract, and 0% germination for seeds exposed to an extract concentration of 0.75% (Fig. 3).

The lowest concentration of leaf extract (0.25%), drove differences in germination probability among regions ($\chi^2_{(1)} = 122.9$, $P < 0.001$; AIC_{null model} = 102.3 vs AIC_{full model} = 215.2). However, there were no differences between the native and non-native ranges in germination probability ($\chi^2_{(1)} = 0.167$, $P = 0.682$; AIC_{null model} = 120.3 vs AIC_{full model} = 118.5). Post-hoc tests revealed that germination in Australia and California extracts was higher than in extracts from the other regions (60%, and 45%, respectively; see Appendix S2 Table S1 for Post-hoc tests, Fig. 3). Leaf-extracts of plants from Turkey (21%), Argentina (19%), and Spain (17%) resulted in stronger germination inhibition; and plants from Chile resulted in the highest germination inhibition (4.0% germination). Germination of seeds exposed to an intermediate concentration of leaf extract (0.5%) was different among regions overall ($\chi^2_{(1)} = 19.14$, $P = 0.001$; AIC_{null model} = 38.65 vs AIC_{full model} = 47.79) and there were differences between the native and non-native ranges too ($\chi^2_{(1)} = 4.569$, $P = 0.032$; AIC_{null model} = 40.71 vs AIC_{full model} = 43.28). Leaf-extracts from Spain resulted in the highest germination (7.4%), which was still lower than any of the

germination rates in the 0.25% concentration. Leaf-extracts from all other regions suppressed germination to <2%, with no seeds germinating when exposed to extracts from plants from Chile and Argentina. However, post-hoc tests showed no significant differences among the groups tested (p -value > 0.05), (see Appendix S2, Table S2 for post-hoc tests results, Fig. 3). At this concentration, non-native populations suppressed germination more than natives (0.7% vs 4.62%), and this was generally determined by populations from Argentina and Chile which had zero germination. Exposure of seeds to the highest leaf-extract concentration (0.75%) completely suppressed seed germination for *C. solstitialis* from all population sources.

Lettuce seeds in both control groups took an average of 2.2 days to germinate after being sown, reaching a peak in the third day (See Appendix S3, Table S1). Seeds exposed to leaf-extract concentrations took longer times to germinate. The number of days to germination for seeds exposed to 0.25% leaf extract was different among regions ($F = 3.091$, d.f. = 5,173; $P = 0.010$, $AIC_{\text{null model}} = 192.3$ vs $AIC_{\text{full model}} = 197.7$), with seeds treated with the Australian extract emerging first (4.7 days $\pm$ 0.21) followed by seeds exposed to Californian, Spanish, Chilean and Turkish extracts (all longer than 5 days) whereas seeds treated with Argentinean extract took the longest to germinate (6.0 days $\pm$ 0.47), (See Appendix S2, Table S3 for post-hoc tests results, Appendix S3, Table S1, Fig. 4). However, no differences were found between the native and non-native ranges ($\chi^2_{(1)} = 2.332$, $P = 0.126$, $AIC_{\text{null model}} = 723.8$ vs $AIC_{\text{full model}} = 724.2$). Seeds exposed to the 0.5% extract showed no differences in days to germination neither among regions ($F = 0.397$; d.f.=3,9; $P = 0.757$, $AIC_{\text{null model}} = 13.84$ vs $AIC_{\text{full model}} = 9.460$), nor between ranges ($\chi^2_{(1)} = 0.226$, $P = 0.634$, $AIC_{\text{null model}} = 57.66$ vs $AIC_{\text{full model}} = 55.88$) (See Appendix S2 Table S4 for post-hoc tests results, Appendix S3 Table S2, Fig. 4).

Effects of leaf-extracts on *Lactuca sativa* radicle length

Control seedlings achieved an average root length of 12.0 ± 0.06 mm (mean $\pm$ SE) for positive control and 9.3 ± 0.04 mm for negative control. Radicle lengths of germinants exposed to 0.25% leaf extracts were much shorter than in the controls, and significantly differed among regions overall ($F_{\text{region}} = 4.091$, $df=5$, 171; $p = 0.001$; $AIC_{\text{null model}} = -421.9$ vs $AIC_{\text{full model}} = -411.9$). No significant difference were found between the native and non-native ranges ($\chi^2_{(1)} = 0.321$, $P = 0.570$; $AIC_{\text{null model}} = 89.08$ vs $AIC_{\text{full model}} = 87.40$). *Lactuca sativa* seeds exposed to 0.25% Australian, Californian and Spanish leaf extracts produced roots that were 60% shorter than those of the controls; and seeds exposed to extracts from Chile, Turkey and Argentina produced roots that were about 80% shorter than the controls (see Appendix S2, Table S5 for post-hoc tests, Fig. 5). The average radicle length of the seeds in the 0.25% extract was 3.6 ± 0.02 mm. Seeds exposed to 0.5% treatment also showed significant differences among regions overall ($F_{\text{region}} = 8.117$, $d.f. = 5, 13$; $p = 0.001$, $AIC_{\text{null model}} = -115.0$ vs $AIC_{\text{full model}} = -98.09$), but there were no differences between the native and non-native ranges ($\chi^2_{(1)} = 1.625$, $P = 0.202$; $AIC_{\text{null model}} = -49.41$ vs $AIC_{\text{full model}} = -49.79$). No seeds exposed to extracts from Argentina or Chile germinated, but root length decreased more than 85% in response to extracts from Australia and Spain, and more than 90% in response to extracts from Turkey and California, compared to the controls (see Appendix S2, Table S6 for post-hoc tests, Fig. 5). Average radicle length was 1.3 ± 0.01 mm for this treatment.

Discussion

Our findings supported the potential allelopathic effects of *C. solstitialis* leaf leachates, and showed substantial variation in leaf-surface chemical composition and inhibitory effects among world regions. Allelopathic effects were strikingly weaker in the non-native regions of California and Australia than in any of the other four studied regions worldwide. Extracts from introduced populations in Chile and Argentina showed strong inhibitory effects, similar to those from native populations in Spain and Turkey. This suggests that *C. solstitialis* may have developed different leaf chemical profiles in different parts of the world where it has been introduced. To the best of our knowledge, this is one of the first studies to report lower allelopathic effects in introduced regions of an invasive compared to native regions (but see Lankau et al. 2009). This suggests that chemical defenses might not be as important for invasive success in these regions as they are in other non-native regions and in the native range, potentially due to shifting selection pressures such as biogeographical differences in the relative importance of competition or herbivore release (Gruntman et al. 2017). However, confirming such a conclusion would require expanding the target species to include those that are dominant in each particular non-native region.

Geographically, leaf-surface extracts of *C. solstitialis* were highly variable in the composition of alkanes, sesquiterpene lactones, and pentacyclic triterpenoids; but with a clear predominance in all regions ($\geq 64\%$) of the biologically important sesquiterpene lactones. Leaf-surface composition of sesquiterpene lactones as a group was similar between regions, but sesquiterpene lactone sub-types differed among regions. Most of the sesquiterpene lactones identified in this study have been shown to possess a wide spectrum of biological activities and

360 to be active at very low concentrations which makes them good candidates as allelopathic
361 compounds. The mode of action of sesquiterpene lactones is not well understood, but we
362 know that they can interfere with cell membrane functions and protein synthesis, and cause
363 oxidative stress (Bachelier et al. 2006). For example, *acroptilin* and *repin* from *C. solstitialis*
364 were found to promote lettuce root elongation at 10 ppm, but inhibit elongation at 80 ppm
365 (Stevens and Merrill, 1985). Both *repin* and *subluteolide*, the major sesquiterpene lactones
366 identified in our study are highly reactive epoxides (Burrows and Tyrll, 2013), with *repin*
367 surpassing the toxicity of *subluteolide* > *janerin* > *cynaropicrin* > *acroptilin* > *solstitialin* in an *in*
368 *vitro* cytotoxicity bioassay (Riopelle and Stevens 1993). *Repin* was also found to inhibit the
369 growth of plants *in vitro* (Stevens et al. 1990) and interfere with insect larval growth and deter
370 insect herbivory (Rosinski et al. 1988). Another class of compounds, *cynaropicrin* derivatives
371 have well-documented antimicrobial and anti-insect activities (Bachelier et al. 2006;
372 Bhattacharyya et al. 1996; Cis et al. 2006) and in this study were found exclusively in non-native
373 regions. Similarly, *solstitialin*, a chemical specific to *C. solstitialis* (Heywood et al. 1977) could
374 potentially represent a novel toxin in the non-native areas, particularly because of its high
375 toxicity against eukaryotic and prokaryotic cells (Cheng et al. 1992). Although both *solstitialin*
376 *A-3 acetate* and *solstitialin A-13 acetate* were present in the leaf extracts, only the latter is
377 known to exhibit biological activity (Özçelik et al. 2009), but its concentrations were higher in
378 native plants relative to non-natives. Lastly, *janerin*, a compound displaying promising insect
379 antifeedant activity (Cis et al. 2006), also exhibited levels similarly high across all regions. Our
380 PCA analysis indicated that each region had somewhat unique concentrations of many of the
381 major potential allelochemicals, such as *solstitialin-A3-acetate* and *solstitialin-A13-acetate* in

Spain and Turkey, *janerin* and *subluteolide* in Australia, *repin* and *cebellin F* in Argentina and California or a unique “cocktail” of different chemicals such as *epoxyrepdiolide*, *acroptilin*, *aguerin B*, and *desacylcynaropicrin* in Chile. Note that our PCA analysis was based on 24 variables (representing all the chemical compounds identified in the extract), and explained 66% of the inertia variance in the first two axes (PC1 and PCA2). For this type of chemical compound analysis it is well known that the more variables, the less explanation in the multivariate ordination. Other studies on plant phytochemical profiles reported similar or lower values of variance for constituents of essential oil of *Dracocephalum kotschyi* (PC1+PC2 = 68%) (Jalaei et al. 2015) and *Citharexylum spinosum* (PC1+PC2 = 60%) (El Ayeb-Zakhama et al. 2017), and for chemical compounds of *Alliaria petiolata* leaves (PC1+PC2 = 58%) (Barto et al. 2010). Sotes et al. (2015) also profiled the leaf chemistry of *C. solstitialis* from four of the six world regions included in our study, but they did not test for allelopathic effects. That previous study found a similar sesquiterpene lactone fraction as described in this study, with the exception of California, where sesquiterpene lactones concentration as a group was higher. We found fewer different alkanes than Sotes et al. (2015), and for the total amount of alkanes we found 74% more in Spain and California, 44% more in Turkey, and 23% more in Chile. These differences could be due to phenological changes in the chemical content of leaves (see Alvarez 2008; Locken 1985; Geppert et al. 1983), since we sampled rosette leaves in pre-reproductive adult plants, whereas Sotes et al. (2015) sampled flowering plants.

We found substantial geographical variation in chemical profiles, but we could not unequivocally connect specific chemical compound concentrations and the effects of extracts on germination and radicle growth rates. This may be because many different chemical

compounds have similar phytotoxic effects or that interactions between different chemicals create effects that are not explained by the individual chemical concentrations. For example, triterpenes can also disrupt cell membranes (Almeida 2002). Moreover, some of the chemical compounds present in the extracts could not be identified (3-11%), although their absorption spectra indicated similarity to lactones, and these may have enhanced or diminished the biological potency of the different extracts. A recent study has found reduced diversity in leaf microbiome in invading *C. solstitialis* plants compared to natives (Lu-Irving et al. 2019). Our plants were subjected to uniform growing conditions and it is unlikely that some extracts were potentiated by the presence of co-extracted bacterial metabolites.

Our study targeted all chemical compounds on the leaf surfaces, but under natural conditions different compounds may have different rates of release and accumulation into the soil and may not act simultaneously. Additionally, we used a target species which is known to be sensitive to phytochemicals at low concentrations, but the response might have varied with other bioassay species. For instance, a 0.5% w/v aqueous extracts of intact green, senesced leaves and combined ground leaves of *C. solstitialis* from Idaho did not affect the germination of *Bromus tectorum*, *Agropyron spicatum*, *Agropyron intermedium*, *Festuca idahoensis* and *C. solstitialis* itself under greenhouse conditions but negatively impacted radicle elongation for all species (Zamora 1984). An important point to consider in allelopathy is the concentration and persistence of the terpenoids in soil. This can be transient and influenced by the chemico-physical properties of the substrate and the microbial communities (Kokalis-Burelle and Rodríguez-Kábana, 2006). For instance, Picman (1987) found that the compound *isovalantolactone* disappeared completely from soil after three months. Another study showed

that *parthenin* can be rapidly degraded in the soil, in the course of a few days, depending on the dose and the experimental conditions (Belz et al. 2009). Similarly, very small quantities of *cnicin* were found in soil infested with *C. maculosa* (Locken and Kelsey, 1987).

The allelochemical concentrations detected in our study may only partially reflect the real field situation where different biotic and abiotic factors come into play. Certain sesquiterpene lactones in *C. solstitialis* were found to vary seasonally in their concentration in plants from California, with an increase in these compounds as the plants matured (bolting and flowering stage), compared to the rosette stage (Alvarez 2008). However, they found no difference in chemical composition and concentration between different plant parts tested (e.g., leaves, stems and flowers) suggesting that they may all contribute to a potential phytotoxicity during plant life and after plant decomposition; and given the high biomass production in this species it may also result in residual allelopathy (Alvarez 2008). In California, *C. solstitialis* has an extended reproductive season, and flowers after the early season native annuals have set seeds (Roche et al. 1994). The plant starts to lose its leaves by early fall and seeds begin to germinate with the arrival of the fall rains (Sheley et al. 1999). This corresponds with the emergence of native and other exotic species, which makes forms of interference possible. In naturally occurring *C. solstitialis* infestations, averaging a density of 240 plants/m², decaying shoot tissue represents 1.38% w/w of the soil sample (Zamora 1984). Soil mixed with ground *C. solstitialis* shoots and roots and supplemented with nutrients showed increasing inhibition of several plant species with increasing residue concentration in the soil (Zamora 1984). Likewise, soil from field plots with natural vegetation, *C. solstitialis* alone and no vegetation showed that litter removal from soil significantly increased the root length of *B.*

tectorum and *C. solstitialis* (Zamora 1984) but it was not clear whether this was due to nutrient deficiency or allelopathic compounds present in the soil.

Conclusions

Our bioassays showed that *C. solstitialis* leaf leachates can have allelopathic effects, but those effects present substantial biogeographical differences. These differences suggest that there can be lower allelopathic investments in some non-native regions where *C. solstitialis* is nonetheless highly invasive, compared to the native regions. We found differences in allelopathic effects among *C. solstitialis* regions, with plants from some of the non-native regions having weaker effects on lettuce germination, days to germination, and radicle elongation than plants from the native regions. The negative effects of leaf extracts from *C. solstitialis* from non-native California and Australia were far lower than for plants from the other regions, with extracts from Chile having *stronger* negative effects than plants from the other regions. Collectively, this suggests that rapid selection on the biochemical signatures of an exotic invasive plant species can be highly region-specific in different regions globally. Alternatively, founding effects due to introductions from different source populations in different world regions might have resulted in inherently different chemical signatures among regions. Our study is among the first to focus on biogeographic differences in the chemistry composition of allelochemicals and allelopathic effects, thereby providing fundamental insights into plant-plant chemical interactions in an invasion context. The difficulties intrinsic to international sampling and the high amounts of leaf tissue needed for chemical extraction are likely the reason why, to our knowledge, no other study has been able to assess biogeographic

variations of allelopathy in more than 2-3 world regions previously. Further studies are now needed to lay emphasis on within region-variation, i.e., within and among populations of each distinct biogeographical region.

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Author's contribution

REI and DM designed the study, SMML and TMVDPM performed the chemical analysis, GS, LAC, ÖE, CJL, KF, JLH, collected plant seeds, REI, SMML, CR, RMC and DM analyzed the data. REI and DM lead the writing of the manuscript. All authors contributed to earlier drafts of this manuscript and gave final approval for publication.

492 **Data accessibility**

493 Additional information on the statistical tests conducted in this study are included in the
494 Supporting Information for this manuscript. Data on the collinearity test is included in
495 Appendix S1, Tukey post-hoc tests may be found in Appendix S2 and the germination indices
496 are included in the Appendix S3.

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Figure legends

Figure 1. World distribution of *C. solstitialis* according to GBIF (yellow-orange dots), and sampled regions (red dots). Map adapted from GBIF (2017)

Figure 2. Plot of Principal Component Analysis scores (centered and standardized PCA) representing the multivariate space in chemical compounds found in six *C. solstitialis* world regions. Blue dots represent native regions, and red dots non-native regions

Figure 3. Effect of two different concentrations of *C. solstitialis* leaf extract on lettuce seed germination. Different letters indicate statistically significant differences among groups ($p < 0.05$). Both positive and negative control had a germination rate close to 100%

Figure 4. Effect of two different concentrations of *C. solstitialis* leaf extract on days to germination. Both positive and negative control took 2.2 days to germinate. No seeds from Chile and Argentina germinated at 0.5% extract

Figure 5. Impact of three different concentrations of *C. solstitialis* leaf extract on lettuce seedling radicle length (mm). Different letters indicate statistically significant groups ($p < 0.05$)

Table 1. GPS coordinates of the *C. solstitialis* populations sampled in this study (WGS84 format)

Pop	Region	Range	Province	Locality	Latitude	Longitude
1	Turkey	native	Denizli	Pinarkent	37.802833	29.19525
2	Turkey	native	Burdur	Burdur	37.616083	30.146167
3	Turkey	native	Denizli	Serinhisar	37.531556	29.300861
4	Turkey	native	Izmir	Beydağ	38.085861	28.215472
5	Turkey	native	Izmir	Bozdağ	38.301361	28.049861
1	Spain	native	Cuenca	Moncalvillo de Huete	40.24159	-2.687453
2	Spain	native	Lleida	L'Espluga-Calba	41.50499	1.005857
3	Spain	native	Burgos	La Horra	41.728801	-3.834349
4	Spain	native	Valladolid	Castroño	41.392459	-5.276957
5	Spain	native	Zaragoza	Sástago	41.408225	-0.289773
1	Argentina	non-native	La Pampa	Paraje El Tropezón, R14	-36.709	-64.831055
2	Argentina	non-native	La Pampa	El Durazno, R14 km 189	-36.700077	-65.391416
3	Argentina	non-native	La Pampa	Rucanelo, R11 y R10	-36.708944	-64.830833
4	Argentina	non-native	La Pampa	Quehué, R 35 y R18	-37.121611	-64.286611
5	Argentina	non-native	La Pampa	Unanue, R35 km 215	-37.559666	-64.2915
1	Chile	non-native	Talagante	Padre Hurtado	-33.570833	-70.855277
2	Chile	non-native	Talagante	El Monte	-33.689444	-71.055277
3	Chile	non-native	Santiago	Maipú	-33.524722	-70.751666
4	Chile	non-native	Santiago	Lo Barnechea	-33.37	-70.429722
1	California	non-native	Sacramento	Folsom	38.64215	-121.17596
2	California	non-native	Solano	Green Valley	38.20954	-122.14631
3	California	non-native	Napa	Napa	38.339041	-122.15467
4	California	non-native	Solano	Vacaville	38.41059	-121.934338
5	California	non-native	Napa	Napa	38.45353	-122.152875
1	Australia	non-native	NSW	Hume, 5km N of Holbrook	-36.677787	147.369684
2	Australia	non-native	NSW	Gundagai	-35.067197	148.108528
3	Australia	non-native	NSW	18 km N of Cudal	-33.22193	148.90875
4	Australia	non-native	NSW	Koorawatha	-34.016784	148.56897
5	Australia	non-native	NSW	Murringo	-34.319168	148.493212
6	Australia	non-native	NSW	Yass	-34.873953	148.908326

Table 2. Major classes of chemical compounds (%), identified in the *C. solstitialis* final leaf-surface extract by GC-MS analysis

Compound (%)	Turkey	Spain	Argentina	Chile	California	Australia
Alkanes	18.34	18.13	14.54	10.18	28.66	14.69
Sesquiterpene lactones	70.95	64.61	76.71	78.26	55.85	78.32
Pentacyclic triterpenoids	3.51	5.39	4.54	4.96	5.64	2.4
Total identified	92.8	88.13	95.79	93.4	90.15	95.41
Total unidentified	6.7	10.89	4.16	5.74	6.75	3.31
Grand total	99.5	99.02	99.95	99.14	96.9	98.72

Table 3. Sesquiterpene lactones identified in the *C. solstitialis* final leaf-surface extract (%) by GC-MS analysis. The only compounds that appear to have clear biogeographical differences are shown in bold

Compound (%)	Turkey	Spain	Argentina	Chile	California	Australia
C ₁₅ H ₁₈ O ₄ desacylcynaropicrin	0.32	0.37	0.79	0.94	0.82	0
C ₁₇ H ₂₂ O ₆ solstitialin A-3 acetate	0.94	0.32	0	0	0	0
C ₁₇ H ₂₂ O ₆ solstitialin A-13 acetate	10.3	7.73	2.02	1.12	0.85	1.62
C ₁₉ H ₂₃ ClO ₇ acroptilin	2.33	3.09	3.28	5.75	3.77	2.46
C ₁₉ H ₂₂ O ₅ aguerin B	0	0.14	0	0.7	0.47	0
C ₁₉ H ₂₂ O ₆ epoxyrepdiolide	9.57	8.16	7.51	19.07	11.59	11.46
C ₁₉ H ₂₂ O ₇ repin	12.22	26.04	31.58	12	12.88	9.31
C ₁₉ H ₂₂ O ₇ subluteolide	26.5	8.28	9.01	14.57	6.4	30.55
C ₁₉ H ₂₂ O ₇ janerin	8.94	9.25	14.11	18.87	8.38	19.85
C ₂₀ H ₂₄ O ₆ cebellin F	0.12	0.14	0	0	0.22	0.1
C ₂₁ H ₂₄ O ₇ cynaropicrin 3-acetate	0	0	7.51	5.94	8.83	2.97
C ₂₁ H ₂₄ O ₇ cynaropicrin 4'-acetate	0	0	0	0	0.16	0
C ₂₃ H ₂₆ O ₈ cynaropicrin 3,4'-diacetate	0	0.1	0.26	0	0.21	0

Table 4. Pentacyclic triterpenoids (%) identified in the *C. solstitialis* final leaf-surface extract per region, by GC-MS analysis

Compound (%)	Turkey	Spain	Argentina	Chile	California	Australia
C ₃₀ H ₅₀ O β-Amyrin	1.29	1.86	1.57	1.46	1.83	0.81
C ₃₀ H ₅₀ O α-Amyrin	1.01	2.55	1.8	2.27	2.56	1.19
C ₃₀ H ₅₀ O taraxasterol	1.21	0.98	1.17	1.23	1.25	0.4

Table 5. Alkanes identified in the *C. solstitialis* final leaf-surface extract per region (%) by GC-MS analysis

Compound (%)	Turkey	Spain	Argentina	Chile	California	Australia
C ₂₅ H ₅₂ (Linear)	0	0	0	0	0.32	0
C ₂₇ H ₅₆ (Linear)	2.1	2.53	1.34	1.63	3.44	1.42
C ₂₉ H ₆₀ (Linear)	6.77	4.14	2.48	5.3	8.41	2.17
C ₂₉ H ₆₀ (Branched)	0	4.26	3.92	0	6.12	3.34
C ₃₁ H ₆₄ (Linear)	2.14	1.6	1.41	3.25	2.12	2.02
C ₃₁ H ₆₄ (Branched)	4.74	4.17	3.91	0	5.88	4.57
C ₃₂ H ₆₆ (Branched)	1.45	0.45	0.39	0	0.8	0
C ₃₃ H ₆₈ (Branched)	0.6	0.98	1.09	0	1.67	1.17

Figure 1.

Figure 2.

Figure 3.

876 **Figure 4.**

885 **Figure 5.**

886