Phytotoxic effects of volatile and water soluble chemicals of
Artemisia herba-alba

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Abstract

We investigated the phytotoxic effects of volatile and water soluble chemicals produced by the shrub *Artemisia herba-alba* Asso. We conducted a number of germination and early seedling growth bioassays on species that co-exist with *A. herba-alba* in natural semiarid plant communities (*Salsola vermiculata* L., *Lygeum spartum* L., *Pinus halepensis* Mill. and *A. herba-alba* itself). In addition, we assessed the phytotoxic effects of a mixture of phenols that were identified in the aqueous extract of *A. herba-alba* on the germination of such species. We found that volatile chemicals inhibited the germination of *P. halepensis* seeds, promoted the growth of *P. halepensis* seedlings and reduced the root biomass of *S. vermiculata* seedlings, while water soluble chemicals promoted the growth of *L. spartum* and *P. halepensis* seedlings. On the other hand, both volatile and water soluble compounds inhibited the germination of *A. herba-alba* seeds, highlighting its strong autotoxic nature. We did not find a phytotoxic effect of phenols identified (catechol, protocatechuic and vanillic acids) on species that co-exists with *A. herba-alba*, although they had a moderate autotoxic effect. Our results suggest that both the volatile and water soluble chemicals might be involved in chemical interactions among plants in semiarid environments.

Key Words

Aqueous extract, Germination, Phenolic compounds, Seedling growth, Semiarid environment, Volatile compounds.

1 Introduction

Many plants species interfere with neighboring plants beyond direct competition for resources, through the production and release into the environment of secondary metabolites (allelochemicals) by leachates, litter decomposition, root exudates and volatilization (Rice, 1984). Allelochemicals can affect other plants directly by reducing seed germination, growth and survival (Rice, 1984) and indirectly by altering soil microbial communities and their effects on soil biogeochemical processes, *e.g.*, nitrification (Castaldi et al., 2009; Wang et al., 2012). However, allelochemicals are often non-specific compounds and allelopathy may also occur within the same species, which is known as autotoxicity (Ruan et al., 2011). Thus, autotoxicity occurs when allelochemicals released by a plant negatively interfere with germination, growth and survival of the same plant species (Friedman and Waller, 1985; Singh et al., 1999). As such, plant-plant interactions mediated by allelochemicals (*i.e.*, allelopathy and autotoxicity) have the potential to shape the composition and structure of the aboveground vegetation (Alías et al., 2006; Herranz et al., 2006).

Many species are known to be potentially allelopathic in semiarid environments (Araniti et al., 2012; Friedjung et al., 2013). Indeed, harsh abiotic factors such as high temperatures, intense solar radiation and water deficit often enhance the production, accumulation and phytotoxicity of allelochemicals (Akula and Ravishankar, 2011; Chen et al., 2012). Furthermore, in those communities, the joint action of multiple stresses might increase the plant's susceptibility to the effects of allelochemicals (Pedrol et al., 2006). It has been argued that allelopathy may be the result of selection against being facilitative plants (van der Putten, 2009). Similarly, autotoxicity can play a relevant role in avoiding the impacts of intraspecific competition for scarce resources (*e.g.*, water)

under harsh environmental conditions (Armas and Pugnaire, 2011). However, autotoxicity has been rarely explored despite its potential adaptive value in those systems.

It has been claimed that plants in arid areas release mostly volatile allelochemicals (*e.g.*, monoterpenes) while water soluble allelochemicals (*e.g.*, phenols) are more common in cool temperate areas (Chou, 1999; Reigosa et al., 1999). In semiarid environments, however, plants are especially rich in phenols because drought-tolerant plants produce and accumulate those compounds to overcome the oxidative damage caused by drought stress (Bautista et al., 2016; Varela et al., 2016). In addition, even though water scarcity might act as a physical limitation to diffusion of water soluble chemicals, in these systems, there is a substantial water redistribution from the bare soil to vegetation patches and other mechanisms (*e.g.*, water input from fog drip; Callaway, 2007; Ludwig et al., 2005) that may increase the water soluble allelochemicals available to vegetation patches. Consequently, the role of water soluble chemicals, and in particular phenols, on allelopathy and autotoxicity in semiarid environments might have been overlooked.

Phenolic compounds are one of the best-known classes of plant allelochemicals (Muscolo and Sidari, 2010). Phenols comprise several groups including simple phenols, phenolic acids, coumarins, tannins and some flavonoids (Li et al., 2010; Muscolo and Sidari, 2010). Mainly, they are introduced into the environment through plant litter decomposition and, to a lesser extent, as leachates of plant parts (Hättenschwiler and Vitousek, 2000). Once the phenols are released, they interact with nutrient cycling and can limit the availability of certain nutrients (*e.g.*, nitrogen and phosphate; Hättenschwiler and Vitousek, 2000; Inderjit and Mallik, 1997). Biological effects of phenolic compounds in target plants range from changes in cell membrane permeability

to effects on plant photosynthesis, which affect the normal growth and development of the entire plant (for an extensive review, see Li et al., 2010).

The objective of this study was to investigate the phytotoxic effects of the volatile and water soluble chemicals produced by an allelopathic shrub of a semiarid plant community. As allelopathic species we selected *Artemisia herba-alba* Asso. (desert wormwood; Mohamed et al., 2010), a dwarf shrub about 30-40 cm tall, widespread in semiarid areas of the Mediterranean Basin, especially in the Iberian Peninsula, North Africa and the Middle East. Aerial parts of *A. herba-alba*, including leaves, young stems and flowers, contain high amounts of phenolic compounds (Bourgou et al., 2016; Khlifi et al., 2013; Younsi et al., 2016). However, although the effects of *A. herba-alba* aqueous extract have been assessed on different target species (Arroyo et al., 2016; Atoum et al., 2006), the phenolic constituents of the aqueous extract seldom have been characterized. On the other hand, the composition of the essential oils of *A. herba-alba* has been largely investigated (Mohamed et al., 2010; Salido et al., 2004; Younsi et al., 2016), although its phytotoxicity through volatilization has not been assessed before (but see Friedman et al., 1977). We hypothesized that i) not only volatile, but also water soluble chemicals produced by *A. herba-alba* have phytotoxic effects on seed germination and early seedling growth. We further hypothesized that ii) aqueous extract of *A. herba-alba* is rich in phenolic compounds and that iii) some of those phenolic compounds (*i.e.*, identified ones) induce phytotoxic activity.

To address the first hypothesis, we conducted germination and growth bioassays to assess phytotoxic effects of volatile and water soluble chemicals on a set of target species. Target species included the shrub *Salsola vermiculata* L., the perennial grass *Lygeum spartum* L. (two dominant species that co-exist with *A. herba-alba* in natural

semiarid plant communities) and *Pinus halepensis* Mill. (a species commonly used to reforest areas that have natural populations of *A. herba-alba*). *Artemisia herba-alba* was also included as target species to test for autotoxicity (Atoum et al., 2006; Friedman and Orshan, 1975). To address the second and third hypotheses, we first quantified the Total Phenolic Content (TPC) of the aqueous extract, and of the leaves and stems of *A. herba-alba* to estimate the fraction that is actually water soluble. Later, some phenolic compounds in the aqueous extract were identified based on High Performance Liquid Chromatography (HPLC), and the potential phytotoxic effects of the phenolic compounds identified were assessed on seed germination of target species. Additionally, the volatile organic compounds (VOCs) in the shoots of *A. herba-alba* were also identified based on Gas Chromatography-Mass Spectrometry (GC-MS).

2 Material and Methods

2.1 Plant Material Collection and Aqueous Extraction

Mature seeds of *A. herba-alba* and *S. vermiculata*, and mature caryopses of *L. spartum* were collected in the field, in 2014, from a natural plant community in the Middle Ebro Valley, NE Spain. This area is characterized by a semiarid climate, and has an average annual precipitation that ranges between 300 and 400 mm year⁻¹. Mean annual temperature is about 15 °C, with a pronounced continentality (data obtained from the Digital Climatic Atlas of Aragón; <http://anciles.aragon.es/AtlasClimatico/>). Seeds of *P. halepensis*, which were collected in 2011, were obtained from a local garden center. Furthermore, aerial parts (*i.e.*, leaves and stems) of *A. herba-alba* were collected from a number of individuals, randomly selected, in the same plant community before the beginning of bioassays. Specifically, aerial parts of *A. herba-alba* used in the aqueous extraction and in the TPC analysis were collected in March 2015, and aerial parts used

in the volatile bioassays and in the VOCs analysis were collected in December 2015. Aerial parts were air-dried at room temperature for 10 d and stored in a cold chamber at 4°C until its utilization. In the drying process, fresh material lost more than 40% of its weight. The dried aerial parts used in the bioassays of the volatile chemicals were ground nearly to powder, by a mechanical mill, prior to the start of the bioassays.

To obtain the aqueous extract of *A. herba-alba*, the dried aerial parts were soaked in demineralized water (100 g l⁻¹) for 24 h at room temperature (15°C-20°C) in total darkness. The solution was filtered, stored in small portions (30 ml) and frozen (-18 °C) until its utilization.

2.2 Determination of the Phytotoxic Effects of Volatile and Water Soluble Chemicals

2.2.1 Seed Germination Bioassays

The phytotoxic effect of the volatile chemicals released from the aerial parts of *A. herba-alba* was determined by assessing seed germination in the four target species (*S. vermiculata*, *L. spartum*, *P. halepensis* and *A. herba-alba*). Seeds were placed on a layer of Joseph filter paper within 10-cm-diameter Petri dishes and were moistened with 5 ml of demineralized water. Aluminum foil containers (3-cm-diameter) with 0.05, 0.1 or 0.5 g (dry weight) of ground aerial parts were then placed into Petri dishes. Ten replicates (*i.e.*, Petri dishes) of *S. vermiculata*, *P. halepensis* and *A. herba-alba* (10 seed each), and *L. spartum* (5 caryopses each) were utilized at each treatment. The controls were performed placing empty aluminum containers within Petri dishes. Petri dishes were hermetically sealed with parafilm. Seeds were set to germinate in a greenhouse under controlled conditions (12 h of light at 23 °C and 12 h of darkness at 18 °C; Table A.1).

The phytotoxic effect of the water soluble chemicals was determined by assessing seed germination in the four target species following the same design mentioned above (Table A.1). In this case, seeds were moistened with 5 ml of aqueous extract diluted to 0.5, 2 or 5 g l⁻¹, and controls were performed leaching seeds with demineralized water. In addition, seeds were set to germinate in a room that had a temperature that ranged between 24 °C in the day and 20 °C at night, and 12 h of light provided by cool light fluorescent tubes (204 μmol s⁻¹ m⁻²). Germination of *A. herba-alba* seeds failed in those conditions and required lower temperatures to succeed. Therefore, *A. herba-alba* seeds were set to germinate in a thermostatic growth chamber that had 12 h of light photoperiod (210 μmol s⁻¹ m⁻²), light period temperature 15 °C and dark period temperature 5°C (Table A.1). The positions of the Petri dishes were changed randomly every few days and the number of germinated seeds (rupture of seed coats and protrusion of the radicle) was recorded daily for 21 d.

The phytotoxic effect of a mixture of pure phenolic compounds was also determined by assessing seed germination in the four target species. Specifically, catechol, protocatechuic and vanillic acids were used for the mixture because they were identified and quantified in *A. herba-alba* aqueous extract (40.6 mg l⁻¹ in 100 g l⁻¹ aqueous extract; 37.2, 9.4 and 2.2 mg l⁻¹ respectively). Seeds were moistened with 5 ml of three dilutions of the phenolic mixture as treatments (0.24, 0.98 and 2.44 mg l⁻¹). The proportion found for each identified compound was maintained when the mixture was performed. Thus, at each treatment, the concentration of each compound was the same as the concentration of such compounds in either 0.5, 2 or 5 g l⁻¹ of *A. herba-alba* aqueous extract. Seeds were moistened with demineralized water in the controls. The number of replicates, number of seeds per treatment and germination conditions was the same as those in the germination bioassay of aqueous extract (Table A.1).

2.2.2 Early seedling growth bioassays

Seeds of *S. vermiculata*, *L. spartum*, *P. halepensis* and *A. herba-alba* were sowed in 8 x 8 x 7 cm plastic pots (60 pots per species and bioassay) that were filled with approximately 100 g of substrate (peat). To maximize the probability of having at least one seedling per pot, multiple seeds were sowed in each pot. Extra seedlings were removed once more than one seedling had emerged in a pot. Exceptionally, two seedlings of *P. halepensis* were left to grow in some pots because germination was low. *Pinus halepensis* seedlings have pivoting roots, with limited lateral development (personal observation) and, consequently, neighboring seedlings had negligible influence. Pot positions were randomized in space when seeds were set to germinate. In the germination period, pots were watered regularly with tap water. After most of the seedlings had become well established (approximately two weeks), treatments were applied.

The phytotoxic effect of the volatile chemicals released from aerial parts of *A. herba-alba* was determined by assessing early seedling growth in the four target species. Seedlings were set to grow in three portable plastic greenhouses (0.29 m³) under a 12 h of natural daylight photoperiod and a temperature that ranged between 20°C and 25°C on clear days (temperature did not differ significantly among greenhouses, $F_{3,24} = 0.061$, $p > 0.05$, data not shown). Fifteen pots of each seedling species were randomly placed into each greenhouse (60 pots per greenhouse) and either, 3, 10 or 30 g of ground aerial parts (equivalent to 0.05, 0.17 or 0.5 g per pot respectively) were placed in a plastic support that hung about 20 cm above the pots. *Artemisia herba-alba* aerial parts were replaced twice in the bioassay. Controls pots ($n = 60$) were randomly placed into a fourth greenhouse without the aerial parts of *A. herba-alba* (Table A.1). The positions of the portable greenhouses and pots were

randomly changed every few days. Pots were watered regularly with tap water. Greenhouses were open for about 10 min when pots were watered.

The phytotoxic effect of the water soluble chemicals of *A. herba-alba* was determined by assessing early seedling growth in the four target species. Specifically, 15 pots per species were watered weekly with 40 ml of aqueous extract diluted to either, 0.5, 2 or 5 g l⁻¹. Occasionally, the treatment was reduced to 30 ml because the substrate was nearly saturated. In those instances, to ensure that the dosages of water soluble chemicals were maintained, the concentrations of the solutions were increased to 0.7, 2.7 and 6.7 g l⁻¹ respectively. Irrigation with tap water remained as control for the rest of pots. All pots were set to grow in a room that had a temperature of 24°C in the day and 20°C at night, and 12 hours of light provided by cool light fluorescent tubes (204 μmol s⁻¹ m⁻²; Table A.1). The positions of the pots were changed randomly every few days. After eight weeks growing, seedlings were dried at 60 °C for 3-4 days, and the biomass of the whole aerial parts and roots of the seedlings was measured.

2.3 Determination of Volatile and Phenolic constituents

2.3.1 VOCs Extraction and GC-MS Analysis

A 7.5-mg sample of powdered aerial parts was resuspended in 1 ml of distilled water and sonicated for 5 minutes. Then, 0.1 ml of the aqueous extract was placed into a 22 ml crimp cap headspace vial and subjected to headspace solid phase microextraction (HS-SPME). A 65 μM PDMS/DVB (Supelco, Bellefonte, PA, USA) fiber was used for the analysis. Pre-incubation and extraction times were performed at 50 °C for 10 and 20 min respectively. Desorption was performed for 1 min at 250 °C in splitless mode. VOCs trapped on the fiber were analyzed by GC-MS using an autosampler COMBI PAL CTC Analytics (Zwingen, Switzerland), a 6890N GC Agilent Technologies (Santa

Clara, CA, USA) and a 5975B Inert XL MSD Agilent, equipped with an Agilent J&W Scientific DB-5 fused silica capillary column (5%-phenyl-95%-dimethylpolysiloxane as stationary phase, 60 m length, 0.25 mm i.d., and 1 mm thickness film). Oven temperature conditions were 40 °C for 2 min, 5 °C min⁻¹ ramp until 250 °C and then held isothermally at 250 °C for 5 min. Helium was used as carrier gas at 1.4 ml min⁻¹ constant flow. Mass/z detection was obtained by an Agilent mass spectrometer operating in the EI mode (ionization energy, 70 eV; source temperature 230 °C). Data acquisition was performed in scanning mode (mass range m/z 35–220). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC-MS (Agilent). Compound identification was based on both the comparison between the MS for each putative compound with those of the NIST 2005 Mass Spectral library and the match to a GC retention time and Mass Spectra custom library generated using commercially available compounds.

2.3.2 Total Phenolic Content and HPLC-UV Analysis

Total Phenolic Content (TPC) of the aqueous extract, leaves and stems of *A. herba-alba* was measured by spectrophotometry following the Folin-Ciocalteu method (Singleton and Rossi, 1965). Three 0.1-g samples of powdered leaves and stems from various individuals were extracted with 50 ml of 70% ethanol and heated at ebullition temperature for 30 min. Ethanol extracts were filtered (filtration funnel with filter n° 2, 40-100 µm), and 0.1 ml of each extract was diluted in 3.4 ml of distilled water before 0.5 ml of Folin-Ciocalteu reagent was added. After 1 min incubation, 1 ml of Na₂CO₃ solution (20%) was added. The resultant solution was mixed thoroughly by mechanical shaking before being heated in a bain-marie at 40 °C for 20 min. On the other hand, three 0.02-ml samples of aqueous extract of *A. herba-alba* were diluted in 3.48 ml distilled water, and the same above-mentioned protocol was followed. The absorbance

of the samples was read by spectrophotometry at 760 nm (Shimadzu UV-160) against the blank test (distilled water). TPC of the samples was estimated based on a calibration curve that was prepared with standard gallic acid (1 to 10 $\mu\text{g ml}^{-1}$) and was expressed as mg of gallic acid equivalents (GAE) per g of plant material.

Phenolic compounds of the aqueous extract of *A. herba-alba* were identified by HPLC. Before the chemical analysis, an acid hydrolysis was performed to obtain free phenolic compounds. Specifically, 0.3 ml of concentrated hydrochloric acid was added to 1.5 ml aqueous extract of *A. herba-alba* in a glass tube, which was heated at 100 °C for 1 h. This solution was filtered through a 1- μm glass-microfiber disc before being injected into the HPLC system. A pre-column Waters μ Bondapak C18 Sentry Guard Cartridge, (10 μm , 3.9 mm x 20 mm) was placed immediately before a Waters column μ Bondapak 18 C (250 mm x 4.6 mm ID cartridge, 10 μm). The phenolic compounds were eluted with a flow rate of 1.5 ml min^{-1} and a linear gradient from 0 to 20% of solvent B (acetonitrile) in solvent A (distilled water with 0.5% glacial acetic acid), followed by 15 min re-equilibration with solvent A. The UV-visible spectrum was recorded at 280 nm (maximum absorbance of phenolic compounds) with a Waters 996 photodiode array detector. The compounds were identified and quantified by comparing the retention time, the absorption spectrum and the peak area to those of a standard.

2.4 Statistical Analyses

Differences in seed germination among treatments were analyzed, for each target species, using generalized linear mixed models (GLMMs) for repeated measures. Treatment and time of measurement were set as fixed factors, while Petri dish was included as random factor. Three moments were considered as time of measurement: 7, 14 and 21 days (d7, d14 and d21 respectively) after the start of the germination

bioassays (except *S. vermiculata* because germination had ended by d14). A binomial distribution of errors was specified in the models. Tukey's *post hoc* honest significant difference (HSD) tests were used to detect significant differences between pairs of treatments.

Differences in the aerial and root biomass of seedlings among treatments in the growth bioassays were evaluated, for each target species, using one-way ANOVA. Tukey's *post hoc* honest significant difference (HSD) tests were used to detect significant differences between pairs of treatments.

Statistical analyses were performed in R.

3 Results

3.1 Effects of Volatile and Water Soluble Chemicals on Seed Germination

Significant effects of the volatile chemicals released from *A. herba-alba* aerial parts were not observed on germination of *S. vermiculata* and *L. spartum* seeds (Table 1, Fig. B.1). However, the volatile chemicals released from the highest amount of *A. herba-alba* aerial parts inhibited germination of *P. halepensis* (Table 1, Fig. 1A). Furthermore, the volatile chemicals released from any amount of *A. herba-alba* aerial parts also inhibited the germination of its own seeds (Table 1, Fig. 1B).

The water soluble chemicals of *A. herba-alba* did not have a significant effect on the germination of *S. vermiculata* and *L. spartum* seeds (Table 1, Fig. B.2). On the other hand, germination of *P. halepensis* seeds differed significantly among treatments (Table 1). Specifically, the intermediate concentration of aqueous extract promoted seed germination compared to the highest concentration of aqueous extract. However, they did not differ significantly from seeds in the control (Figure 2A). With respect to

germination of *A. herba-alba* seeds, there was an interaction between treatment and time of measurement (Table 1). Overall, the highest concentration of aqueous extract inhibited the germination of *A. herba-alba* seeds at all three times considered (d7, d14 and d21), whereas the lowest and intermediate concentrations only inhibited its germination from d7 (Fig. 2B).

The mixture of pure phenolic compounds (*i.e.*, catechol, protocatechuic and vanillic acids) did not have a significant effect on the germination of *S. vermiculata*, *L. spartum* and *P. halepensis* seeds (Table 1, Figs. B.3 and 3A). However, the highest concentration of the mixture significantly inhibited germination of *A. herba-alba* seeds (Table 1, Fig. 3B).

3.2 Effects of Volatile and Water Soluble Chemicals on Early Seedling Growth

After 8 weeks, the volatile chemicals released from the intermediate and the highest amount of *A. herba-alba* aerial parts significantly reduced the root biomass of *S. vermiculata* seedlings ($F_{3,45} = 11.96$, $p < 0.001$), but non-significant effects were observed on the aerial biomass ($F_{3,45} = 1.64$, $p = 0.19$; Fig. 4). Conversely, the volatile chemicals did not have a statistically significant effect on the root biomass *L. spartum* seedlings ($F_{3,56} = 2.5$, $p = 0.07$), but aerial biomass differed significantly among treatments ($F_{3,56} = 3.84$, $p = 0.01$). Specifically, aerial biomass was lower in *L. spartum* seedlings exposed to the volatile chemicals released from the intermediate amount of ground aerial parts than it was in those exposed to the lowest amount. However, they did not differ significantly from seedlings in the control (Fig. 4). In *P. halepensis*, aerial and root biomass were significantly higher in seedlings exposed to the highest amount of ground aerial parts of *A. herba-alba* than they were in seedlings in the control ($F_{3,49} = 3.03$, $p = 0.04$ and $F_{3,49} = 5.12$, $p = 0.004$; Fig. 4). At all three treatments, the volatile

chemicals of *A. herba-alba* aerial parts did not have a significant effect on the aerial ($F_{3,54} = 1$, $p = 0.4$) or the root biomass ($F_{3,51} = 1.74$, $p = 0.17$) of *A. herba-alba* seedlings (Fig. 4).

The effects of the aqueous extract of *A. herba-alba* on early seedling growth of *S. vermiculata* and *A. herba-alba* could not be quantified because mortality was very high and too few seedlings survived to the end of the bioassay (data not shown). The aerial biomass of *L. spartum* seedlings watered with the highest concentration of aqueous extract was significantly higher than the aerial biomass of seedlings in the control ($F_{3,55} = 2.72$, $p = 0.05$), but seedling root biomass did not differ significantly among the treatments ($F_{3,55} = 2.11$, $p = 0.11$; Fig. 5). In addition, both the aerial and root biomass were significantly higher in the seedlings of *P. halepensis* irrigated with either the intermediate or the highest concentration of aqueous extract than they were in the control seedlings ($F_{3,53} = 4.16$, $p = 0.01$ and $F_{3,52} = 4.29$, $p = 0.009$; Fig. 5).

3.3 Volatile and Phenolic Constituents

The GC-MS analysis allowed the identification of 49 volatile constituents, accounting for 92.58 % of total VOCs (Table A.2). Among the identified compounds, oxygenated monoterpenes were the most abundant (43.58 %), followed by sesquiterpene hydrocarbons (37.24 %) and monoterpene hydrocarbons (10.9 %). Only a small amount of oxygenated sesquiterpenes were found (0.4%). Specifically, 1,8 cineole (8.7%), camphor (6.9%) and *cis*-chrysanthenyl acetate (14.9%) were found to be major constituents, accounting for two thirds of the oxygenated monoterpenes. The major sesquiterpene hydrocarbon was δ -cadinene (10.5%). α -pinene (3.25%) and *p*-cymene (2.6%) accounted for over a half of the monoterpene hydrocarbons (Table A.2).

Chemical analysis determined that the TPC of *A. herba-alba* leaves (54.25 ± 1.15 mg GAE g⁻¹) and stems (36.15 ± 0.48 mg GAE g⁻¹) were approximately four and three times higher, respectively, than the TPC in the aqueous extract of *A. herba-alba* (12.39 ± 0.36 mg GAE per g of plant material used in aqueous extraction).

The HPLC chromatogram at 280 nm indicated that the aqueous extract of *A. herba-alba* contained at least 15 different phenolic compounds, one of which was very abundant. However, only three of the compounds could be identified and quantified in the aqueous extract: catechol, protocatechuic and vanillic acids (37.2, 9.4, and 2.2 mg l⁻¹ respectively; Fig. 6).

4 Discussion

In this study we investigated the phytotoxic effects of the volatile and the water soluble chemicals produced by the shrub *A. herba-alba* on seed germination and early seedling growth of species that co-exist with *A. herba-alba* in natural semiarid plant communities, including *A. herba-alba* itself. Overall, our results showed that both the volatile and water soluble chemicals strongly inhibit the germination of *A. herba-alba* seeds, highlighting the autotoxic nature of this species. Previous studies also found autotoxic effects in *A. herba-alba*. Atoum et al. (2006) reported that exposure to aqueous extract obtained from *A. herba-alba* fruits might reduce initial elongation of *A. herba-alba* seedlings. In addition, it was found that water soluble chemicals can increase the mortality of *A. herba-alba* seedlings that emerge from the seed bank near *A. herba-alba* individuals (Arroyo et al., 2016). In a field study, Pueyo *et al.* (2016) found a pattern of segregation between adult *A. herba-alba* individuals and other adults, juveniles or seedlings of *A. herba-alba*. In the same line, Friedman and Orshan (1975) found that although *A. herba-alba* seeds are dispersed in close proximity to the parent

plants, only few seedlings are established under their canopy. Our results suggest that the volatile and the water soluble chemicals may drive autotoxicity of *A. herba-alba* during germination, which might lead to the observed pattern among *A. herba-alba* individuals in natural plant communities.

Autotoxicity has been observed in many crop systems (Singh et al., 1999), but also in natural environments such as Mediterranean forest of *P. halepensis* (Fernandez et al., 2008) and shrublands of *Cistus ladanifer* L. (Alías et al., 2006) and *Cistus clusii* Dunal (Foronda et al. *in rev.*). However, its ecological meaning is still not fully understood. It has been suggested that autotoxicity is the consequence of chemical defenses against herbivores and pathogens, or that it forces higher seed dispersal distances, favoring the colonization of new areas and avoiding inbreeding (Nilsen, 2003; Reigosa et al., 1999; Singh et al., 1999). Alternatively, given that individuals of the same species occupy a similar niche, a strong competition for resources might be expected to occur among them (Armas and Pugnaire, 2011). Therefore, in semiarid environments where resources are scarce (*e.g.*, water), autotoxicity may arise as an adaptive advantage to regulate population density, diminishing self-recruitment near parent species by inhibiting or delaying germination until rainfall and, thereby, preventing intraspecific competition (Armas and Pugnaire, 2011; Friedman and Waller, 1985; Singh et al., 1999). Indeed, autotoxicity (or biochemical recognition sensu Renne et al., 2014) might be a common strategy to avoid competitive environments (Renne et al., 2014). Thus, autotoxicity might play a more important role than previously thought in determining the structure and dynamics of allelopathic shrub species in semiarid environments.

The composition of the essential oils of *A. herba-alba*, has been thoroughly investigated (Mohamed et al., 2010). Overall, oxygenated monoterpenes are often found

to be the major constituents of the volatile fraction. In particular, the presence of α/β -thujones, camphor, chrysanthenone and 1,8-cineole is commonly reported in essential oils of *A. herba-alba* (Bourgou et al., 2016; Mohamed et al., 2010; Salido et al., 2004; Younsi et al., 2016). In agreement with previous studies on the chemical composition of essential oils of *A. herba-alba* from Spain (Feuerstein et al., 1988; Salido et al., 2004), we found large amounts of 1,8 cineole and the absence of thujones. However, it was unusual the presence of nonanal. Many volatile constituents of *A. herba-alba*, such as α/β -pinene, camphor and 1,8 cineole, has been reported as phytotoxic (Martino et al., 2010). However, the major volatile compound in our sample, *cis*-chrysanthenyl acetate (16.52%), has not been reported as phytotoxic yet. Altogether, our results underscore the potential of *A. herba-alba* litter as a significant source of volatile phytotoxic compounds involved in its autotoxicity.

We also found an important total amount of phenolic compounds in the leaves and stems of *A. herba-alba*. Some studies have reported TPC values over 100 mg GAE g⁻¹ in *A. herba-alba* (Khelifi et al., 2013) but others have reported values that were lower than those in our study (Bourgou et al., 2016; Younsi et al., 2016). The variation in TPC among studies might be caused by differences in the plant organs used in the analyses, in the season of plant material collection, in climatic conditions in different regions and in the extraction procedure (Khelifi et al., 2013). Similarly, phenolic compounds including catechol, protocatechuic and vanillic acids were abundant in the aqueous extract of *A. herba-alba*. Protocatechuic and vanillic acids were already reported in *A. herba-alba* (Bourgou et al., 2016; Seddik et al., 2010). Our results showed that the pure phenolic mixture inhibited germination of *A. herba-alba* seeds, thus, suggesting that identified phenols might have contributed to the net autotoxic effect of the aqueous extract of *A. herba-alba*. However, autotoxic effects of the aqueous extract were

apparently stronger than the effects of the mixture of pure phenols. On the one hand, there were other compounds in the aqueous extract of *A. herba-alba* that were unidentified, and might have contributed more to its autotoxicity than did the identified phenols. In this sense, it should be noted that the HPLC chromatogram at 280 nm indicated the presence of an extraordinarily abundant unidentified compound (peak 7, Fig. 3). *Artemisia herba-alba* aerial parts are known to contain other phenolic compounds such as chlorogenic, ferulic and caffeic acids, apigenin, luteolin and quercetin (Saleh et al., 1985; Seddik et al., 2010; Younsi et al., 2016). In addition, it could be possible that there were very small amounts of certain compounds belonging to other chemical classes (*e.g.*, terpenes) in the aqueous extract (Weidenhamer et al., 1993), which might have also inhibitory effects. On the other hand, pure compounds in the mixture probably degrade faster than they do in the aqueous extract (Li et al., 2015).

On the other hand, our results showed limited phytotoxic effects of the volatile and water soluble chemicals produced by *A. herba-alba* on the germination of neighboring plant species. However, water soluble chemicals from *A. herba-alba* had positive effects on early seedling growth of *L. spartum* and *P. halepensis*. Low concentrations of phenolic acids such as those identified in the aqueous extract of *A. herba-alba* can induce stimulatory responses (Einhellig, 2004). Similarly, we found that the volatile chemicals of *A. herba-alba* had a positive effect on the early seedling growth of *P. halepensis*. In line with our results, Tsubo *et al.* (2012) found that exposure to low concentrations of the volatile chemicals released by *Artemisia adamsii* Besser stimulated photosynthetic rates in the grass *Stipa krilovii* Roshev., and, hence, its growth. It is likely that those species have some kind of tolerance to the allelochemicals produced by *A. herba-alba*. A long associational history between allelopathic plants (and their allelochemicals) and other coexisting species might lead some species to

become adapted to its chemical neighbor (Ehlers and Thompson, 2004; Mallik and Pellissier, 2000). Our results suggest that in such cases a positive effect of volatile and water soluble chemicals on plant growth might be expected. In fact, it has been found that, in semiarid plant communities of the Middle Ebro Valley, *A. herba-alba* may act as a nurse plant facilitating the establishment of *L. spartum* and other perennial grasses regardless of its phytotoxicity (Arroyo et al., 2015; Pueyo et al., 2016). The ratio of susceptible and tolerant species will dictate the impacts of an allelopathic species at community level. Consequently, phytotoxic effects should be assessed from a multispecific perspective, including a number of target species covering several life forms and abundances in order to get reliable data on the impacts of an allelopathic species on plant community organization.

In conclusion, our study underscored that both the volatile and the water soluble chemicals might be involved in intra- and inter-specific interactions in semiarid environments. We found only limited phytotoxic effects of the volatile chemicals released from *A. herba-alba* on the germination of some neighboring plant species (*P. halepensis*) and generalized positive effects of the volatile and water soluble chemicals on early seedling growth. These findings suggested that target species selected in our study might have a certain tolerance to phytotoxic compounds of *A. herba-alba* because of co-evolution. On the other hand, both types of chemicals had strong autotoxic effects on the germination of *A. herba-alba* seeds. Furthermore, we found that phenolic compounds were abundant in the *A. herba-alba* aqueous extract. Our results emphasized that the identified phenolic compounds can be partially responsible for the autotoxicity of the aqueous extract, pointing out the potential role that these chemicals can play in semiarid environments. Further bioassays should use living donor plants and incorporate soil microorganisms in order to obtain more realistic evidences about the

significance of allelopathy in natural conditions. In addition, more research is required to fully understand the significance of autotoxicity in semiarid environments and its ecological meaning.

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Table 1 Result of GLMM analyses for the effect of treatment and time of measurement on seed germination of target species. Significance of the change in deviance was tested by comparing models with and without the effect of each term, assuming a χ^2 distribution. Significant effects ($p < 0.05$) are highlighted in bold

Species	Variable	Volatile chemicals				Aqueous extract				Phenolic mixture			
		Res. Deviance	χ^2	df	<i>p</i> -value	Res. Deviance	χ^2	df	<i>p</i> -value	Res. Deviance	χ^2	df	<i>p</i> -value
<i>S. vermiculata</i>	Treatment	223.37	7.43	3	0.06	257.58	2.17	3	0.54	253.28	2.07	3	0.56
	Time	230.79	0.01	1	0.92	259.64	0.11	1	0.74	255.23	0.11	1	0.73
	Treatment:Time	223.34	0.02	3	0.99	257.30	0.17	3	0.98	253.12	0.05	3	0.99
<i>L. spartum</i>	Treatment	463.05	1.16	3	0.76	336.93	0.87	3	0.83	313.55	1.02	3	0.80
	Time	281.52	182.7	2	< 0.001	289.34	48.46	2	< 0.001	271.01	43.56	2	< 0.001
	Treatment:Time	273.76	6.62	6	0.36	281.11	7.37	6	0.29	267.41	2.62	6	0.85
<i>P. halepensis</i>	Treatment	849.99	22.02	3	< 0.001	652.20	11.66	3	< 0.01	369.20	6.95	3	0.07
	Time	355.90	516.12	2	< 0.001	405.91	257.94	2	< 0.001	300.26	75.89	2	< 0.001
	Treatment:Time	330.68	5.23	6	0.51	385.94	8.26	6	0.22	290.31	3.07	6	0.80
<i>A. herba-alba</i>	Treatment	263.91	27.27	3	< 0.001	566.72	37.53	3	< 0.001	552.56	15.81	3	< 0.01
	Time	271.60	19.58	2	< 0.001	382.80	221.45	2	< 0.001	343.23	225.14	2	< 0.001
	Treatment:Time	243.74	0.57	6	0.99	329.77	15.07	6	0.02	317.60	9.53	6	0.15

Figure legends

Fig. 1 Germination (mean % $\pm$ SE) of A) *P. halepensis* and B) *A. herba-alba* seeds exposed to volatile chemicals released from 0.05, 0.1 and 0.5 g of ground *A. herba-alba* aerial parts. Different letters indicate significant differences among treatments (Tukey's HSD test)

Fig. 2 Germination (mean % $\pm$ SE) of A) *P. halepensis* and B) *A. herba-alba* seeds moistened with 5 ml of *A. herba-alba* aqueous extract diluted to 0.5, 2 and 5 g l⁻¹. Different letters indicate significant differences among treatments (Tukey's HSD test)

Fig. 3 Germination (mean % $\pm$ SE) of A) *P. halepensis* and B) *A. herba-alba* seeds moistened with 5 ml of a mixture of catechol, protocatechuic and vanillic acids. At each treatment, the concentration of each compound was the same as the concentration of such compounds in either 0.5, 2 or 5 g l⁻¹ of *A. herba-alba* aqueous extract. Different letters indicate significant differences among treatments (Tukey's HSD test)

Fig. 4 Dry biomass (mean $\pm$ SE) of aerial parts (dark grey) and roots (light grey) of seedlings of *S. vermiculata*, *L. spartum*, *P. halepensis* and *A. herba-alba* (from left to right) after two months of exposure to either 0.05, 0.17 or 0.5 g per seedling of ground *A. herba-alba* aerial parts. Different letters among treatments indicate significant differences (Tukey's HSD test)

Fig. 5 Dry biomass (mean $\pm$ SE) of aerial parts (dark grey) and roots (light grey) of seedlings of *L. spartum* (left side) and *P. halepensis* (right side) after two months of watering with either 0.5, 2 or 5 g l⁻¹ of aqueous extract of *A. herba-alba*. Different letters among treatments indicate significant differences (Tukey's HSD test)

674 **Fig. 6** HPLC profile of *A. herba-alba* aqueous extract. Numbered peaks indicate the
675 different phenolic compounds detected in aqueous extract at 280 nm. Only three
676 compounds could be identified by comparing retention time, absorption spectrum and
677 the peak area to those of a standard

678

Fig. 1

Fig. 2

687 **Fig. 3**

689 **Fig. 4**

Fig. 5

Fig. 6