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

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A B S T R A C T

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.

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

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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 A1).

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 A1). 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 A1). 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