Weeds for weed control: Asteraceae essential oils as natural herbicides

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Abstract

The aim of this experiment was to test some weed species as a source of natural herbicides. In this perspective, the botanic family of Asteraceae was selected. Twenty Asteraceae species were collected during flowering time and evaluated in terms of essential oil (e.o.) yield and quality (inhibition of germination and growth of weeds). Half species showed a sufficient e.o. yield (from about 0.1 to 1.43%) to test these phytochemicals *in vitro* as germination inhibitors of two typical weeds: *Amaranthus retroflexus* and *Setaria viridis*. In spite of the higher resistance the latter weed, the concentration of 100 µg L^{-1} of e.o. of the two *Artemisia* species and *Xanthium strumarium* e.o. was able to totally inhibit germination. Moreover, at 50 µg L^{-1} the same e.o. showed full inhibition of *A.retroflexus* seeds. The comparison of their effectiveness at sub-optimal doses allowed a further selection (halving) of the most promising e.o. sources. After their chemicals characterization, they were tested as post-emergence herbicide on seedlings of the same weeds. Their spraying at different concentrations (10, 100 and 1000 mg L^{-1}) during two different phenological stages of weed seedlings (cotyledons and the third true leaf), showed the best performances for the e.o. of *Artemisia annua* and *X. strumarium*. In particular, the e.o. of the latter species, were then tested again on both weeds to monitor the dynamics of plant injury, showed a reduction of plant fresh weight (about 20-30% after 10 days) and chlorophyll content (totally destroyed, after the same time), confirming their total and rapid effectiveness. Finally, a discussion about the agronomic context of the possible application of these natural herbicides was carried out.

Running title: natural herbicides

Highlights

► Some wild Asteraceae species showed satisfactory essential oil content.
► Their weed seed germination-inhibition ability evidenced possible interest as natural herbicides.
► Their use as herbicide on weed seedling confirmed this agronomic use.
► This herbicide action through new physiological pathway could have crucial importance.
► Low-impact agroecosystems and urban environment appears be the ideal targets of these natural herbicides

Keywords
Weed control; allelopathy; Bioassay; new crops.

Introduction

The growing need to make possible agricultural productivity in a context of environmental sustainability has stimulated research in the study of cropping systems less dependent on pesticides of synthetic origin (Tilman et al., 2002). In this frame, the weed control strategies plays a crucial role since their conventional management imply the use of a wide range of herbicides. On the other hand, it is well known that they typically involve a strong environmental impact on both terrestrial (Freemark and Boutin, 1995) and acquatic wildlife (Fleeger, 2003). Indeed, such environmental contamination has detrimental effect on biodiversity loss (Relyea, 2005) and their ecological functionality (Hooper et al., 2005). It is also known that the biological complexity has crucial importance on both ecosystem stability (Tilman et al., 2006) and long-term agroecosystem productivity (Paoletti et al., 1992). These agro-environmental requirements have stimulated the search for alternatives to the employment of conventional herbicides through mechanical (Van der Weide et al., 2008), physical (Ascard, 1998), agronomic (Teasdale, 1996), and biological (Muller-Scharer et al., 2000) strategies. On the other hand, if conventional herbicides are not used, the economic sustainability of cropping systems becomes highly vulnerable (Bond and Grundy, 2001). As a result, there is an increasing demand to optimize the already available agronomic strategies (Hatcher and Melander, 2003) and even to discover new natural herbicides (Duke et al., 2000) capable of allowing an appreciable and eco-friendly weed management (Ahluwalia, 2007).
In this context, toxins extracted from fungi and other microorganisms were tested (Li et al., 2003), as well as other secondary metabolites from higher plants (Dayan et al., 2012), to evaluate their impact on the invasiveness of the surrounding vegetation (Macías et al., 2001). This phenomenon,

known as "allelopathy" (Weston and Duke, 2003), is based on the release of phytochemicals by live or dead tissue (Qasem and Foy, 2001) capable of a herbicide-like action (Putnam, 1988).

In this frame, essential oils play a physiological action as allelochemicals, and consequently they are good candidates as potential bioherbicides (Dudai et al., 1999).

Interesting results were shown both in terms of inhibition of germination (Angelini et al., 2003) and growth (De Almeida, 2010), confirming a generalized biological action that implies plant toxicity (Bakkali, 2008).

It is important to note that even these natural substances are not exempt from risks of toxicity for man (Hoagland et al., 2007). However, essential oils, although so closely dependent on the ingested amount (Smith et al., 2005), are commonly added to foods (Burdock and Carabin, 2009) due to their antibacterial properties (Burt, 2004). Their usefulness for humans is confirmed by their use as medicaments (Edris, 2007). Furthermore, their biodegradability is reassuring in terms of food safety of an agro-ecosystem protected by weeds using essential oils. On the contrary, the most criticality is represented by the economic aspect (Auld and Morin, 1995) since their cost is usually high for both the inputs needed for the cultivation of crops and their low yields (Sangwan et al., 2001).

The use of essential oils in agriculture for crop protection could be economically viable if they were extracted from plants which are characterized not only by high e.o. yields, but also by high productivity of biomass, such as *Eucalyptus,* whose e.o. has already been tested as a natural pesticide (Singh et al., 2005; Batish et al., 2008). The possibility of using the e.o. produced by common aromatic crops, i.e. origan, basil and thyme, seems to be not affordable because of the high cultivation costs.

On the contrary, the use of herbaceous plants appears a more feasible possibility due to their lower environmental requirements.

Since many Asteraceae species are widely spread as a pioneer plant in natural, agricultural, and even urban ecosystems (Benvenuti, 2004), they could represent an economic plant biomass for extraction of these allelochemicals (Vyvyan, 2002), often produced in quite high amounts (Chon et al., 2003).

The aim of this study was to test the possible herbicidal activities ("*in vitro*" and "*in vivo*") of essential oils extracted from some Asteraceae species widely distributed in the various Mediterranean environments.

Material and methods

Plant material

During the spring- summer periods of the years 2007-2010 flowersheads of 20 different Asteraceae species were collected in different areas of Tuscany (Table 1). The criterion for determining the time of collection has been uniformed with the phenological stage of full flowering (May-June, depending on the species).

To evaluate the potential biomass that could be obtained from each species in cultivation, areas colonized mostly exclusively by only one species were localized. The biomass of this monospecific vegetation was evaluated after the flowersheads harvest from a square meter areas (5-10 replication for each species).

The plant material was submitted to two different drying procedures: i) dried in a ventilated heater (set to 50°C) for 1-2 days in order carry out the biomass evaluation and ii), dried in the air, in the dark, at room temperature (about 25° C) for the essential oils extraction.

Essential oil extraction and analysis

The essential oils were obtained by hydrodistillation of the dried ground material in a Clevenger-like apparatus for 2 h.

The yield of the essential oils was calculated per unit area, i.e. by using the values of dry biomass of the collected flowersheads from 1 m^2 areas.

The GC analyses were accomplished with an HP- 5890 series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60 °C for 10 min, ramp of 5 °C/min to 220 °C; injector and detector temperatures, 250 °C; carrier gas helium (2 mL/min); detector, dual FID; split ratio, 1:30; injection, 0.5 μL of a 10% hexane solution.

GC-EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240°C respectively; oven temperature programmed from 60°C to 240°C at 3°C/min; carrier gas helium at 1 ml/min; injection of 0.2 μl (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against

commercial (NIST 2014 and ADAMS 2007) and home-made library mass spectra built up from pure substances and components of known oils and MS literature data (Swigar and Silverstein, 1981; Davies, 1990; Adams, 2007; Joulain and König, 1998).

"In vitro" weed germination test

In order to evaluate the effect of essential oils on the germination of weed seeds, two common species (one broadleaf and one grass) typically widespread in the several agroecosystem were selected: *Amaranthus retroflexus* and *Setaria viridis*. These tests were carried out using the essential oils of the only 10 Asteraceae species that showed a higher yield.

The seeds of these two species were placed in 15 cm diameter Petri dishes (50 seeds each) lined with a filter paper (Whatman no. 1) suitably moistened with 7 cm^3 of distilled water. Each essential oil was added to Petri dishes (assayed by micropipettes) on the surface of small glass containers (3 cm) lens-shaped, without any upper cover in order to permit the diffusion of the vapors inside the sealed (parafilm) Petri dishes, avoiding direct contact with the seeds. The tested essential oil quantities were 10 and 100 µg L^{-1}; in addition, controls consisting of distilled water, were performed. The capsules were then incubated at 25°C in climatic chambers equipped with fluorescent tubes (THL PHILIPS 20W/33) producing white light (about 100 mol m^{-2} s^{-1}), using a 12h/12h photoperiod.

The number of germinated seeds was evaluated every 2 days (radicle appearance) until no further emergence was observed (after one week).

"In vivo" toxicity test

This analysis was carried out for the most active 5 essential oils screened by the above "*in vitro*" tests.These assays were carried out on seedlings emerged on alveolar containers (30 x 50 cm) with holes of 4 cm in diameter (6 cm depth, for a total of 60 seedling per container), preliminarily filled with a common peat substrate, widely used in nursery crops.

Seedling of *Amaranthus retroflexus* and *Setaria viridis* were obteined by sowing 2-3 seeds per hole and leaving only one seedling after emergence. They were placed under the same climatic conditions of the above-mentioned germination tests. During incubation, sub-irrigation was carried out maintaining the soil moisture in the ideal conditions for seed germination and seedling emergence. At the appearance of the first true leaves, the seedlings were sprayed with an

aqueous solution of essential oils using a surfactant (Tween® 80, 1% V/V) as emulsifier. The doses distributed on the plants by means of a micro airbrush, simulated a hypothetical distribution in post-emergence of the essential oils. During this distribution, the spray has been shielded by overturned plastic glass (pierced at the base to the nozzle insertion). In such way the desired dose was entirely convey on a known surface. The tested essential oils concentrations were of 0 (distilled water and surfactant), 10, 100 e 1000 mg L^{-1}. The volume of each concentrations was standardized to 30 g m^{-2}, simulating the practical use of a common not selective herbicide such as glyphosate (Baylis, 2000).

The visual criterion of toxicity evaluation of the essential oils was the following: •= absent or negligible, ••= evident, but followed by resilience, •••= total (not followed by regrowth).

The tests were repeated 3 times (each on the same alveolar containers with 60 seedlings each) for each of the two weeds, during two different growth stages (cotyledon/s and true leaf) for each of the 5 essential oils, for a total of 60 containers (2 species x 5 e.o. x 2 phenological stages x 3 replicates).

Toxicity dynamics

The essential oil showed the highest efficacy in the "*in vivo*" test was selected for further experiments to evaluate the toxicity dynamics. The plants were grown as above, with the only difference that in this case alveolar containers with larger holes (7 cm diameter) were used in order to grow 24 plants per container for longer time and without any plant-plant undesired interferences. After 2 weeks from weed sowing, the e.o. was sprayed as above described using a single dose of 1000 mg L^{-1}. After 1 , 5 and 10 days from treatment, the plants were cut and, together with untreated controls, immediately weighed. The test was replicated 3 times for a total of 36 containers: 2 weeds x 3 times from treatment x 2 treatment (e.o. + untreated control) x 3 replicates. For the evaluation of the toxicity dynamics, a chlorophyll loss test was performed on leaf tissue disks (1 cm^2) taken from each plant treatment (1, 5 and 10 days) on treated and untreated weed seedlings. Extraction was carried out in N,N-dimethylformammide, and concentration was determined on a unit area basis of chlorophyll a and b calculated from the absorbance (spectrophotometer SHIMADZU Mod.UV-1204) of the extract at 664 and 647 nm, respectively, according to Moran (1982).

Statistical analyses

For all the experiments (germination and post-emergence toxicity tests), a completely randomized experimental design was adopted. After testing for homogeneity of variance, all percent data (germination test) were arc sin-transformed. Angular values and untransformed data (values not expressed as percentages) were subjected to analysis of variance (ANOVA) using the Student Newman–Keuls test ($p<0.05$ and/or $p<0.01$) for separation of means. For the statistical analyses, commercial software (CoHort software, Minneapolis, MN, USA) was used.

Results

Essential oil yield

Table 2 shows the e.o. production of the 20 Asteraceae species. As can be observed, eight species (*Carlina corymbosa*, *Centaurea cyanus*, *Centaurea solstitialis*, *Cirsium arvense*, *Coleostephus myconis*, *Picris echioides*, *Scolymus hyspanicus* e *Senecio vulgaris*) showed only negligible e.o. yields. On the contrary, *Artemisia annua* evidenced the highest e.o. content, reaching a 1.43% yield. Lower quantities, but still satisfactory, were recorded in descending order by *Artemisia verlotorum* (0.40%), *Otanthus maritimus* (0.37%), *Achillea millefolium* (0.35%). Intermediate results, just above 0.1%, were shown by *Xanthium strumarium* (0.13%) and *Helicrysum italicum* (0.11%). The remaining species gave lower yields, below 0.1%, such as *Anthemis cotula* (0.07%), *Inula viscosa* (0.06%), *Helianthus tuberosus* (0.05%), *Pulicaria dysenterica* (0.03*%), Bidens tripartita* (0.02%) and *Conyza canadensis* (0.02%).

However, these e.o. yields, express only partially the attitude to the e.o. production since it must also be considered the flowersheads biomass produced per unit area. Indeed, *X.strumarium*, in spite of its poor e.o. production, showed the highest flowerheads productivity (278 g m^{-2} of dry biomass), thus achieving an e.o. yield of 0.36 g m^{-2}. This double evaluation (e.o. concentration and flowerheads biomass) evidenced appreciable performances, in terms of e.o. yield per unit area, for *A.annua* (2.55 g m^{-2}) and *A.verlotiorum* (0.87 g m^{-2}). Other satisfactory yields were shown by *A.millefolium* (0.32 g m^{-2}) and *O.maritimus* (0.25 g m^{-2}). On the contrary, *I.viscosa* (0.10 g m^{-2}), *H.italicum* (0.10 g m^{-2}), *H.tuberosus* (0,08 g), *A.cotula* (0,07 g m^{-2}), *B.tripartita* (0.03 g m^{-2}), *C.canadensis* (0.02 g m^{-2}) and *P.dysenterica* (0.01 g m^{-2}) gave clearly scarce e.o. yields.

The remaining species, showed very low e.o. production, and consequently not appreciable yields per unit area. Therefore they were excluded by the successive tests.

"In vitro" seed germination inhibition

The various e.o. showed a broadly diversified effect in germination (Table 3). Even at the lower dose (10 µg L^{-1}), all the e.o. exerted a dramatic and statistically significant ($p < 0.05$) germination inhibition of *Amaranthus retroflexus* seeds. In comparative terms, a lesser efficacy was observed for the e.o. obtained from *H. tuberosus*, *B. tripartita*, *H. italicum*, *I. viscosa* and *A. cotula*. Nonetheless, they were able to inhibit about 75% of germination with respect to the control. The highest germination inhibition was shown, in the some weed species, by the e.o. extracted from *A. millefolium*, *O. maritimus* that was able to reduce germination to only 6 and 5% respectively. An excellent inhibition performance was shown by the e.o. of. *A. annua*, *A. verlotiorum* and *X. strumarium,* able to completely prevent the germination of *A. retroflexus* seeds. At this lower dose, *S. viridis* were the least sensitive to e.o. exposition. In the case of the e.o. obtained from *B.tripartita* and *H.tuberosus* the germination was statistically similar ($p<0.05$) to the control. However, the gradient of effectiveness of the different e.o. was similar to that observed against *A.retroflexus*, although less marked. Also in this case, the e.o. of *A.verlotiorum*, *A.annua* and *X.strumarium* showed the greatest inhibition power since the germination of the control (77%) was reduced to 35, 32 and 22%, respectively. Intermediate results (39-60%) were shown by the e.o. of the other Asteraceae species. At the highest dose (100 µg L^{-1}), the germination of *S.viridis* was completely inhibited by the e.o. of *A.annua* and *X.strumarium*. The same dose of the e.o. of *A.verlotiorum*, *A.cotula* and *O.maritimus* showed an appreciable, but incomplete inhibition (18, 21 and 24%, respectively). The e.o. of the remaining Asteraceae had a suboptimal ihibition on *S.viridis* (33-50%).

In the case of the more sensitive *A.retroflexus,* only the e.o. of *O.maritimus* and *B.tripartita* allowed a very poor germination (7 and 5% respectively). All the other e.o. showed a complete inhibition of germination at this concentration.

In summary, the most promising "*in vitro*" results indicated the best inhibition performances for the e.o. of *A.annua*, *X.strumarium* and *A.verlotiorum,* followed by those of *A.millefolium* and *O.maritimus*. The other e.o. shown a marked lesser degree of interest, at least against these two weeds. Consequently, they have not been taken into consideration in further experiments.

Essential oils composition

Table 4 shows the chemical composition of the most agronomically interesting essential oils extracted from the various Asteraceae species. Their composition is very heterogeneous among the various species. Despite their complexity, in each species some chemicals are well represented.

In *A.millefolium*, the main constituents are artemisia ketone (25.3%), *trans*-pinocarveol (20.9%), camphor (12.9%), β-thujone (5.3%), viridiflorol (4.3%) and borneol (3.6%). In *A.annua* a marked prevalence of 1,8-cineole (23.4%), *trans*-sabinyl acetate (12.5%), artemisia ketone (12.4%), camphor (10.4%) and α-pinene (7.0%) was detected.

The other species belonging to the same genus, *A. verlotiorum*, in addition to chrysanthenone (22.2%), is characterized by similar amount of 1.8-cineole (19.4%) and about half amount of camphor (4.8%). Other volatiles that characterize this species are β-pinene (16.3%), 2,6-dimetyl phenol (4.1%), and β-caryophyllene (4.0%). In *O.maritimus* prevails camphor, which reaches 33.6%. Other chemicals found in this species were yomogi alcohol (18.6%), artemisia alcohol (16.3%) and artemisyl acetate (5.8%). Finally, in *X.strumarium* the main chemicals are borneol (30.3%), isobornyl acetate (12.2%), camphene (11.8%), limonene (11.6%) and tricyclene (6.9%). Generally, the main constituents belong to the chemical class of oxygenated monoterpenes, ranging from 54.6% in *A.verlotiorum* to 83.7% in *O.maritimus*. Monoterpenes hydrocarbons were found in much lesser amounts. These chemicals were detected mainly in *A.verlotiorum*, *A.annua*, *O.maritimus* and *A.millefolium* (22.7, 15.7, 13.2 and 8.6%, respectively). In *X.strumarium* they reached the highest percentage (26.8%). The other chemical classes are poorly represented, with the exception of sesquiterpene hydrocarbons (10.0%) in *A.verlotiorum*.

"In vivo" weed toxicity

Already at the lower dose (10 mg L^{-1}), the essential oils of *A. annua* and *X .strumarium* showed an evident toxicity, albeit followed by resilience, in both tested weeds. However, this was shown only during the most sensitive cotyledon phenological stage (Table 5). This toxicity was maintained even in the next third true leaf stage, but only in the case of the e.o. of *X. strumarium*. At the higher dose of 100 mg L^{-1}, all the e.o. showed, at the cotyledon stage, a total toxicity following their distribution on *A.retroflexus* seedlings. This full effect, due to the more sensitive initial growth stage, was also observed on seedlings of *S. viridis* but only when treated with the e.o. of *A. annua* and *X. strumarium*. Only the latter e.o. was still fully effective during the next third true leaf stage. In all other cases, the toxicity was followed by resilience (regrowth from damaged tissues) in both weeds.

These doses, often sublethal, allowed a valid comparison tool for the evaluation of the e.o. toxicity. Indeed, the highest dose (1000 mg L^{-1}) showed a full efficacy in spite of their Asteraceae origin, weed species and its phenological stage.

In summary, the e.o. of *X. strumarium* showed the most interesting results for its higher toxicity, already detected at the lower doses, even during the less sensitive phenological stage of the weeds. Consequently, the following tests aimed to study the toxicity dynamics of these potential natural herbicides, of this e.o.

Figure 1 shows the phytotoxicity dynamics (fresh weight and chlorophyll content) in *A. retroflexus* and *S.viridis* seedlings after treatment with the e.o. of *X. strumarium*.

Both weeds experienced, after just 5 days from treatment, a clear (and statistically significant, $p < 0.05$) fresh weight decrease. In *A. retroflexus* seedlings (Fig. 1A), fresh weight was reduced from about 0.4 g plant^{-1} to less than 0.3 g plant^{-1}. Similarly, *S.viridis* seedlings (Fig. 1B) reduced their fresh weight from about 0.3 g plant^{-1} to less than 0.2 g plant^{-1}. After 5 days from treatment, both weeds showed a further collapse of their fresh weight that was reduced, in both cases, to about 0.1 g plant^{-1}. This treatment also led to a drastic and sudden chlorophyll loss. In both species, after 5 days, the chlorophyll content was only about a quarter of the initial value. After another 5 days, chlorophyll was completely destroyed and seedlings appeared, other than well dried, even without any green pigmentation due to the disappearance of chlorophyll.

Discussion

Essential oil yield

As expected, the various Asteraceae species showed a very variable e.o. content (Table 2).

Unfortunately, almost half of the tested species has no any interest as e.o. source since eight of them produced only trace amounts. In addition to these eight species, further two were also discarded (*C.canadensis* and *P.dysenterica)* because of both scarce e.o. and flowersheads productivity. On the contrary, it was very encouraging the e.o. richness of *A.annua* (1.43%), especially considering its non-domesticated origin. Its yield could also reach 4% depending on the environmental conditions and the chemotype, as already reported by other authors (Holm et al., 1997). Only this species reached e.o. yields comparable to those of typical aromatic crops such as those belonging to the botanical family of Lamiaceae, i.e. *Satureja hortensis* (about 2%, Bahler et al., 2002), *Lavandula* spp. (2-9%, Renaud et al, 2001) and *Origanum vulgare* (2.5-4%, Azizi et al., 2009).

Even if no species reached such levels, it should be noted that the tested wild species have the advantage of poor agronomic requirements, and consequently their cultivation could be economically sustainable.

The other species belonging to the genus *Artemisia* (*A.verlotiorum*) showed interesting e.o. content, confirming yields comparable to other reported for the Mediterranean environment (Vernin, 2000).

Despite of the satisfactory e.o. content of *O.maritimum* (0.37%) and *A.millefolium* (0.35%), the third species having a promising productivity was *X.strumarium*. Indeed, in spite of its not very high e.o. content (0.13%), mainly located in leaves (Esmaeili et al., 2006), its lower yield is counterbalanced by the larger production of flowersheads biomass. On the basis of these results, the experimental interest has therefore focused on half of the initially selected. Consequently, for the subsequent *"in vitro"* toxicity tests, only the ten most promising species (e.o. and flowersheads biomass production) were used.

"In vitro" weed germination test

As expected, a wide range of germination inhibition of was found. This diversified action was elicited not only as a function of the e.o. source, but also in terms of sensitivity of the two tested weeds, with *S.viridis,* lesser sensitive than *A.retroflexus*. However, despite this diversified sensitivity of the two weeds, the e.o. of *X.strumarium* showed a marked inhibition starting from the lower dose, revealing to be a very promising herbicide. Albeit less effective, even e.o. of *A.annua* and *A.verlotiorum* showed an appreciable effect in spite of the *S.viridis* higher tolerance.

Under an agronomic point of view, the e.o. of the these three Asteraceae confirmed a strong attitude as germination inhibitors. However, albeit to a lesser extent, an appreciable efficacy was shown also by the e.o. of *O.maritimus* and *A.millefolium,* even at the lower dose (10 µg L^{-1}), on the more sensitive *A.retroflexus.* In addition, the higher dose (100 µg L^{-1}) allowed an appreciable inhibition even against the more tolerant *S.viridis*.

On the basis of these results, the further "*in vivo*" experiments were conducted by halving the number of species tested as e.o. source. These insights were carried out on both: i) chemicals identification of the five most interesting e.o. sources and ii) test of these phytochemicals as natural herbicides against of the same weed species.

Chemicals of the most agronomically promising essential oils.

It is not easy to establish, on the complexity of the chemical composition, the components responsible of the biological activity. However, the richness in limonene of the e.o. of *X.strumarium* appears closely linked with its excellent performance. Indeed, such marked

phytotoxicity of this monoterpenes hydrocarbon was already observed in *Amaranthus viridis* (Vaid et al., 2011). Probably, the simultaneous presence of borneol, an oxygenate monoterpene, has a crucial role in enhancing such action since these compounds, previously characterized in Lamiaceae aromatic crops, was already found to have a marked inhibition activity in both weed and crops germination (Angelini et al., 2003). This confirmed the crucial role of monoterpenes, already found to be elicitors of germination inhibition (Martino et al., 2010). These compounds were found capable to affect energy metabolism (Singh et al., 2002) and consequently the plant allelopathic performance (Duke, 2003). Perhaps, even monoterpenes hydrocarbons could play a crucial role since these compounds were found at high percentages in the e.o. of this species. However, in comparative terms, oxygenated monoterpenes are more effective than monoterpenes hydrocarbons as germination inhibitors in both weeds and crops (Vaughn and Spencer, 1993). Probably, the mix of different chemical classes could have a synergistic effect. For example, the coexistence of 1,8-cineole and α-pinene (oxygenated monoterpene and monoterpene hydrocarbon, respectively), relatively abundant in *A.annua*, could be the elicitors of its significant inhibition performance. On other hands, the phytotoxicity of cineole derivatives and of several other monoterpenes, appears to be associated with the presence of an epoxide ring (Dayan et al., 2012).

The hypothesis of the crucial role of monoterpenes is supported by previous studies that reported the oxygenated monoterpene artemisia ketone as the main responsible of the inhibitory effect in spite of the diversified activity against different weeds, such as *Lantana camara* and *Amaranthus hybridus* (Verdeguer et al., 2009).

On the other hand, the e.o. of the taxonomically related *A.verlotiorum* showed a weaker action, which could results from the lower concentration of oxygenated monoterpenes. However, both *Artemisia* species, typically rich in terpenoids *(*Ahmad and Misra, 1994), showed a strong germination inhibition on both weeds.

Other oxygenates monoterpenes, such as camphor (abundant in *O.maritimus*), artemisia ketone and *trans*-pinocarveol (abundant in *A.millefolium*), in spite of their relatively lower activity, appears to be the main phytoxic compounds of the oils of *O.maritimus* and *A.millefolium*.

In vivo weed toxicity

The dose of 1000 (mg L^{-1}) of e.o. fully confirmed the hypothesis that these chemicals may constitute interesting natural herbicides. At this concentration, each of the five e.o. completely

devitalized the two weeds regardless of their phenological stage. However, in order to be able to carry out a comparison between the different e.o. sources, the dose able to exert the optimal herbicidal performance, was found to be of crucial importance. Indeed, the lower doses, together with the less sensitive phenological stages, give the best information of the comparative assessment of the various e.o.. The sub-lethal dose of only 10 (mg L^{-1}) appears to be a good reference for the to carry out a comparative evaluation.

Only in the cases of *X.strumarium* and *A.annua* e.o., even at this concentration they showed a partial seedling toxicity at the cotyledon stage (weed injury followed by resilience) and this address particular interest towards these two Asteraceae species. However, in the subsequent third true leaf stage, such toxicity is maintained exclusively by the e.o. of *X.strumarium*.

This strong toxicity, fully confirms its excellent performance already observed in the *in vitro* germination tests. This correspondence between inhibition of germination and growth suggests that the biological action of e.o. is generalized and do not have a determined site of action. This was already interpreted as loss/distruption of mitotic activity capable of reduction/inhibition of both germination and seedling growth (Singh et al., 2005).

At the highest dose of 100 (mg L^{-1}) e.o., all the e.o. showed a total (*A.retroflexus*) or partial (*S.viridis*) effectiveness on seedlings, at the early cotyledon stage, demonstrating a growth-stage mediated plant-sensitivity. During the less sensitive phenological stage (third true leaf), only e.o. of *X. strumarium* maintained its effectiveness, confirming to be the most interesting species as natural herbicides source. Although it is not possible to determine which is the most active chemical, probably the phytotoxicity may be attributed, as mentioned above, to monoterpenes, whose *in vitro* toxicity has been previously reported (Brown, 1987). In particular limonene, very abundant in *X.strumarium,* has already shown strong germination and growth inhibition in both weeds (Vaid et al., 2011) and crops (Ibrahim et al., 2004). Consequently, this species was chosen for further studies focused on the evaluation of toxicity dynamics. The spraying with an e.o. solution (1 g m^{-2} of 1000 mg L^{-1}) resulted in a rapid (5 days) and significant ($p < 0.05$) fresh weight decrease in both weeds, with a contemporaneous collapse of the seedling architecture (Fig. 1). After further 5 days, the seedlings became even more dry and completely unable of any chance of resilience. The simultaneous whitening of the leaf tissues indicates the destruction of the chlorophyll molecules and, probably, of chloroplast integrity.

While it is not yet fully clear the physiological mechanism of the e.o. toxicity, it was found that they inhibit the mitochondrial respiration (Abrahim et al., 2003) and are even able to damage the

membrane integrity, which further affects pH homeostasis and equilibrium of inorganic ions (Lambert et al., 2001).

Conclusions

The hypothesis that the e.o. extracted from common weeds may have herbicidal effects was fully confirmed. Although an extensive literature is available on their generalized biocidal effect for the crop protection by weeds, microorganisms and insects (Isman, 2000; Murray, 2000), these substances are mainly derived from aromatic crops and their economic profile is the major agronomic restraint. This experiment demonstrated that these natural pesticides can also be obtained from common weeds.

The main advantage of their hypothetical cultivation as crops dedicated to the extraction of natural herbicides, appears represented by the easy mechanization of the flowerheads harvesting operations due to their herbaceous (non-woody) nature, simple by mowing. In addition, it appears crucial to point out that their wild germplasm implies poor agronomic requirements for their hypothetical cultivation. Consequently, although the costs to obtain the e.o. from these Asteraceae is at present not exactly definable, it is very likely that they may constitute a promising biodiversity resource for the obtaining of relatively cheap natural herbicides.

In summary, these results are very encouraging for assuming a hypothetical category of new crops dedicated to an environmentally friendly weed control. These natural herbicides, could to be useful in both organic cropping agroecosystems and even in the case of conventional agriculture since their action through different and multiple mechanisms of action (Duke et al., 2000b) could minimize the evolution of herbicide resistance due to repeated use of classic products (Powles and Yu, 2010).

Finally, it is perhaps in the urban ecosystem that these substances may constitute the most important agronomic innovation since such environment requires particular safe products in terms of human health.

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2 367-389.
3

Asteraceae species	Environments of plant collection	Locality	Geographic coordinates
Achiellea millefolium L.	Mountain pasture	Cutigliano (PT)	44° 11’ N, 10° 76’ E
Anthemis cotula L.	Dry meadow	S.Piero (PI)	43° 67’ N, 10° 34’ E
Artemisia annua L.	Wet meadow	Asciano (PI)	43° 74’ N, 10° 45’ E
Artemisia verlotiorum L.	Abandoned field	Agnano (PI)	43° 73’ N, 10° 47’ E
Bidens tripartita L.	Agroecosystem	Asciano (PI)	43° 74’ N, 10° 45’ E
Carlina corymbosa L.	Rocky meadow	Agnano (PI)	43° 73’ N, 10° 47’ E
Centaurea cyanus L.	Emmer wheat field	Camporgiano (LU)	44° 15’ N, 10° 34’ E
Centaurea solstitialis L.	Dry meadow	Agnano (PI)	43° 73’ N, 10° 47’ E
Cirsium arvense (L.) Scop.	Agroecosystem	Sansepolcro (AR)	43° 57’ N, 12° 12’ E
Coleostephus myconis (L.) Rchb.	Abandoned field	Cecina (LI)	43° 32’ N, 10° 51’ E
Conyza canadensis (L.) Cronq.	Peri-urban areas	San Giuliano (PI)	43° 75’ N, 10° 43’ E
Helianthus tuberosus L.	Field margin	Asciano (PI)	43° 74’ N, 10° 45’ E
Helichrysum italicum (Roth) G. Don.	Abandoned quarry	Agnano (PI)	43° 73’ N, 10° 47’ E
Inula viscosa (L.) Aiton	Abandoned field	San Giuliano (PI)	43° 75’ N, 10° 43’ E
Otanthus maritimus (L.) Hoffmanns. & Link	Hind dune	Marina di Bibbona (PI)	43° 23’ N, 10° 52’ E
Picris echioides L.	Roadsides	Asciano (PI)	43° 74’ N, 10° 45’ E
Pulicaria dysenterica (L.) Bernh.	Dry meadow	Agnano (PI)	43° 73’ N, 10° 47’ E
Scolymus hispanicus L.	Sandy meadow	Follonica (GR)	42° 92’ N, 10° 77’ E
Senecio vulgaris L.	Agroecosystem	Asciano (PI)	43° 74’ N, 10° 45’ E
Xanthium strumarium L.	Agroecosystem	Asciano (PI)	43° 74’ N, 10° 45’ E

Table 1. Geographical and ecological information about the localities of collection of the different plant species used as essential oil sources.

Species source of E.O.	Dry biomass of flowerheads (g m^{-2})	Plant richness in E.O. (%)	Essential oils yield (g m^{-2})
Achiellea millefolium	93	0.35	0.32
Anthemis cotula	108	0.07	0.07
Artemisia annua	179	1.43	2.55
Artemisia verlotiorum	219	0.40	0.87
Bidens tripartita	105	0.02	0.03
Carlina corymbosa	63	tracks	-
Centaurea cyanus	76	tracks	-
Centaurea solstitialis	64	tracks	-
Cirsium arvense	124	tracks	-
Coleostephus myconis	81	tracks	-
Conyza canadensis	98	0.02	0.02
Helianthus tuberosus	159	0.05	0.08
Helichrysum italicum	88	0.11	0.10
Inula viscosa	175	0.06	0.10
Otanthus maritimus	68	0.37	0.25
Picris echioides	69	tracks	-
Pulicaria dysenterica	56	0.03	0.01
Scolymus hispanicus	68	tracks	-
Senecio vulgaris	79	tracks	-
Xanthium strumarium	278	0.13	0.36

Table 2. Essential oils production per unit area (g m^{-2}) of the 20 Asteraceae species as a function of their flowerheads biomass (g m^{-2}) and richness (%).

Weed	Plant source of E.O.	Concentration (µg L^{-1})		
		0	10	100
		Germination %		
Amaranthus retroflexus	*Achillea millefolium*	85 a	6 c	0 d
	Anthemis cotula		15 b	5 d
	Artemisia annua		0 d	0 d
	Artemisia verlotiorum		0 d	0 d
	Bidens tripartita		22 b	7 d
	Helianthus tuberosus		24 b	0 d
	Helicrysum italicum		19 b	0 d
	Inula viscosa		18 b	0 d
	Othanthus maritimus		5 c	0 d
	Xanthium strumarium		0 d	0 d
Setaria viridis	*Achillea millefolium*	77 a	49 b	18 d
	Anthemis cotula		51 b	21 d
	Artemisia annua		32 c	0 e
	Artemisia verlotiorum		35 c	19 d
	Bidens tripartita		74 a	50 b
	Helianthus tuberosus		72 a	55 b
	Helicrysum italicum		59 b	35 c
	Inula viscosa		60 b	33 c
	Othanthus maritimus		55 b	24 d
	Xanthium strumarium		22 d	0 e

Table 3. Germination inhibition induced by two different doses (µg L^{-1}) of essential oils, from 10 different Asteraceae species, on *Amaranthus retroflexus* and *Setaria viridis* seeds. Means followed by different letters show statistical difference ($p<0.05$) within species.

1

Compounds	L.r.i.[1]	C.c.[2]	Plant source of essential oils				
			Achillea millefolium	*Artemisia annua*	*Artemisia verlotiorum*	*Otanthus maritimus*	*Xanthium strumarium*
			%				
(E)-2-hexenal	854	nt			0.4		
santolina triene	908	mh	0.7	0.1		0.3	
α-thujene	931	mh	0.3	0.3	0.2	0.3	0.6
tricyclene	934	mh	0.8			0.3	6.9
α-pinene	939	mh		7.0	2.5	0.6	
camphene	953	mh	2.4	3.1	0.4	7.6	11.8
sabinene	977	mh	1.2	2.9	0.6		1.8
β-pinene	980	mh	1.2	1.3	16.3	0.3	5.4
myrcene	991	mh				3.8	
dehydro-1,8-cineole	992	om	0.1			0.4	
mesitylene	996	nt	0.1		0.5		
yomogi alcohol	998	om	1.5	0.8		18.6	
α-phellandrene	1005	mh			1.1		
α-terpinene	1018	mh	0.6	0.6	0.5		
p-cymene	1026	mh	1.1	0.2	1.0		0.3
limonene	1031	om	0.6	0.3	0.1		11.6
1,8-cineole	1033	om	1.5	23.4	19.4	0.1	
artemisia ketone	1063	om	25.3	12.4	0.5	0.3	
cis-sabinene hydrate	1069	om	1.2	0.4	0.8		0.3
artemisia alcohol	1084	om	1.2	2.3		16.3	
eucarvone	1084	om				0.2	
terpinolene	1089	mh	0.3	0.2	0.1		
α-pinene oxide	1095	om		0.2	0.9		0.3
linalool	1099	om	1.0			0.4	
trans-sabinene hydrate	1101	om	0.6				0.3
2,6-dimethyl phenol	1102	nt			4.1	0.7	
α -thujone	1103	om	0.4				
(Z)-3-hexenyl propanoate	1106	nt		0.4			
β-thujone	1115	om	5.3				
deydro sabina ketone	1118	om		0.6			
cis-*p*-menth-2-en-1-ol	1122	om	0.2	0.1			0.5
chrysanthenone	1123	om			22.2	4.4	
α-campholenal	1126	om		0.2			0.4
trans-pinocarveol	1140	om	20.9	3.5			1.6
camphor	1144	om	12.9	10.4	4.8	33.6	1.5
β-pinene oxide	1156	om		1.5			
sabina chetone	1158	om		0.3			
pinocarvone	1163	om	0.5	2.4	0.6		1.3
borneol	1166	om	3.6	0.4	1.3	0.2	30.3
artemisyl acetate	1173	om				5.8	
4-terpineol	1178	om	2.1	1.8	0.8		0.7
myrtenol	1194	om	0.5	2.1			1.8
verbenone	1204	om			1.8		
trans-pulegol	1215	om	0.2				
trans-carveol	1217	om	0.1			1.7	0.2
cis-carveol	1230	om				0.2	0.2
isobornyl formate	1233	om	0.4		0.3		0.2
cumin aldehyde	1240	om	0.2				
carvone	1242	om				0.5	0.7

cis-chrysanthenyl acetate	1262	om				0.5	
trans-carvyl acetate	1262	om				0.3	
perilla aldeide	1275	om			1.1		
isobornyl acetate	1286	om		1.4		0.2	12.2
trans-sabinyl acetate	1291	om		12.5			
thymol	1293	om		0.2			
cyclosativene	1369	sh		0.2			
α-copaene	1376	sh		0.4	0.2		
β-cubebene	1390	sh					
β-caryophyllene	1418	sh		0.2	4.0		
trans-α-bergamotene	1439	sh				0.1	
α-humulene	1454	sh			0.7		
alloaromadendrene	1461	sh	0.3				
(E)-β-farnesene	1478	sh		0.7	0.3		
germacrene D	1480	sh	0.1	1.7	3.5		0.2
β-selinene	1484	sh		0.1	0.8		0.3
valencene	1492	sh			0.5		0.4
trans-β-guaiene	1501	sh					0.3
trans-γ-cadinene	1513	sh					0.3
isoitalicene epoxide	1515	os	0.1				
ledol	1565	os	0.2				
caryophyllene oxide	1581	os			0.5		1.5
viridiflorol	1590	os	4.3				
Humulene epoxide II	1607	os					0.6
1,10-di-*epi*-cubenol	1614	os	0.2				
T-cadinol	1640	os				0.2	
β-eudesmol	1649	os				0.4	0.7
selin-11-en-4-ɣ-ol	1652	os	0.1				
α-cardinol	1654	os	0.2		0.3		
1-tetradecanol	1676	nt	0.1				
(Z)-nerolidol acetate	1678	os	0.3				
cis-α-santalol	1682	os			0.2		
Others compounds[3]			5.1	3.4	6.7	1.7	4.8
Total			100	100	100	100	100
Chemical classes		mh	8.6	15.7	22.7	13.2	26.8
		om	80.3	77.2	54.6	83.7	64.1
		sh	0.4	3.3	10.0	0.1	1.5
		os	5.4	0	1.1	0.6	2.8
		nt	0.2	0.4	5.0	0.7	0

1 Linear retenction indices (HP-5 column).

2 Chemicals classes: mh= monoterpenes hydrocarbons, om= oxygenated monoterpenes; sh= sesquiterpenes hydrocarbons; os= oxygenated sesquiterpenes, nt= not terpenes; pp=phenylpropanoids.

3 Less than 0.1% and/or unidentified compounds.

Table 4. Chemical composition of the essential oils obtained from the five selected Asteraceae species.

Weed	Plant source of E.O.	Weed phenological stage	Concentration (mg L^{-1})		
			10	100	1000
Amaranthus retroflexus	*Achillea millefolium*	Cotyledons	•	•••	•••
	Artemisia annua		••	•••	•••
	Artemisia verlotiorum		•	•••	•••
	Othanthus maritimus		•	•••	•••
	Xanthium strumarium		••	•••	•••
	Achillea millefolium	Third true leaf	•	••	•••
	Artemisia annua		•	••	•••
	Artemisia verlotiorum		•	••	•••
	Othanthus maritimus		•	••	•••
	Xanthium strumarium		•	•••	•••
Setaria viridis	*Achillea millefolium*	Cotyledons	•	••	•••
	Artemisia annua		••	•••	•••
	Artemisia verlotiorum		•	••	•••
	Othanthus maritimus		•	••	•••
	Xanthium strumarium		••	•••	•••
	Achillea millefolium	Third true leaf	•	••	•••
	Artemisia annua		•	••	•••
	Artemisia verlotiorum		•	••	•••
	Othanthus maritimus		•	••	•••
	Xanthium strumarium		••	•••	•••

Table 5. volume distribuito 1 mg-l.

Plant toxicity: •= absent or negligible, ••= evident but followed by resilience, •••= total.

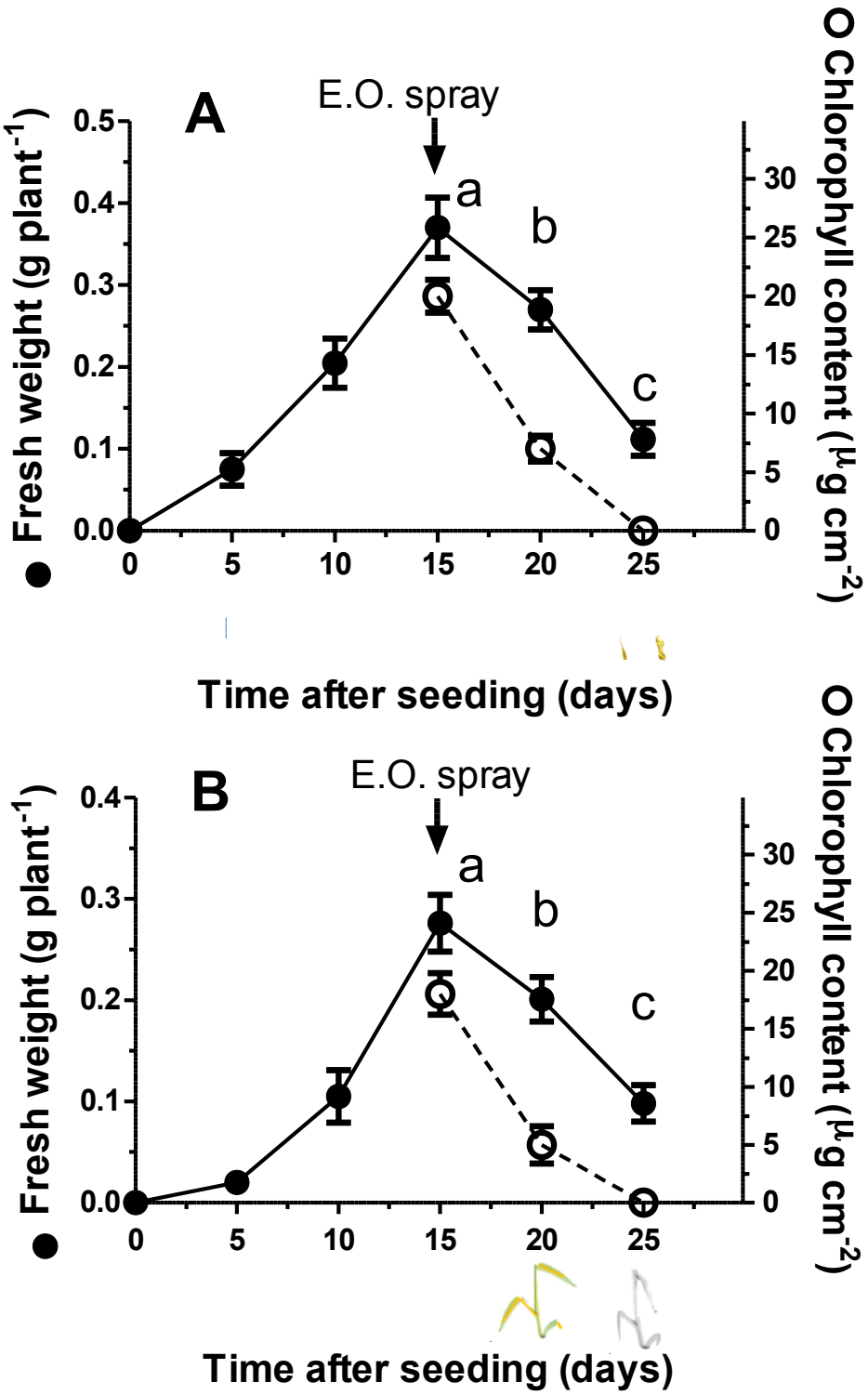

Figure 1. Effect of *Xanthium strumarium* E.O. (30 g m^{-2} of 1000 mg L^{-1} solution) on growth (filled circles) and chlorophyll content (empty circles) of *Amaranthus retroflexus* (A) and *Setaria viridis* (B). Vertical bars represent ± standard errors of the means. The means within both parameters (fresh weight or chlorophyll content) followed by different letters are statistically different ($p<0.05$).