



**Citation:** M. Fernández-Aparicio, A. Cimmino, G. Soriano, M. Masi, S. Vilariño, A. Evidente (2021) Assessment of weed root extracts for allelopathic activity against *Orobanche* and *Phelipanche* species. *Phytopathologia Mediterranea* 60(3): 455-466. doi: 10.36253/phyto-12917

**Accepted:** October 6, 2021

**Published:** December 30, 2021

**Copyright:** ©2021 M. Fernández-Aparicio, A. Cimmino, G. Soriano, M. Masi, S. Vilariño, A. Evidente. This is an open access, peer-reviewed article published by Firenze University Press (<http://www.fupress.com/pm>) and distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Competing Interests:** The Author(s) declare(s) no conflict of interest.

**Editor:** Maurizio Vurro, National Research Council, (CNR), Bari, Italy.

## Research Papers

# Assessment of weed root extracts for allelopathic activity against *Orobanche* and *Phelipanche* species

MÓNICA FERNÁNDEZ-APARICIO<sup>1,\*</sup>, ALESSIO CIMMINO<sup>2</sup>, GABRIELE SORIANO<sup>2</sup>, MARCO MASI<sup>2</sup>, SUSANA VILARIÑO<sup>3</sup>, ANTONIO EVIDENTE<sup>2,\*</sup>

<sup>1</sup>Institute for Sustainable Agriculture-CSIC, Avda. Menéndez Pidal sn, 14004 Córdoba, Spain

<sup>2</sup>Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte S. Angelo, Via Cintia, 80126 Naples, Italy

<sup>3</sup>ALGOSUR S.A., Ctra Lebrija-Trebujena km 5.5, Lebrija – Sevilla, Spain

\*Corresponding author. E-mail: monica.fernandez@ias.csic.es; evidentente@unina.it

**Summary.** Broomrapes (*Orobanche* and *Phelipanche* species) are holoparasitic weeds that infect roots of crop hosts from Asteraceae, Brassicaceae, Apiaceae, Fabaceae, and Solanaceae. The parasitic weeds are difficult to control selectively without crop damage once attached to their roots. Identification of natural compounds with herbicidal activity against pre-attached broomrape stages can provide control alternatives. With the aim to identify plant species with efficacy for broomrape control, organic and residual aqueous phase extracts from roots of seventeen weed species common in south Spanish broomrape-infested fields were assessed as potential inducers of suicidal broomrape germination and as inhibitors of broomrape radicle growth. Assessments were carried out *in vitro* using seeds and seedlings of four noxious broomrape species, *Orobanche crenata* Forsk., *Orobanche cumana* Wallr., *Orobanche minor* Sm. and *Phelipanche ramosa* (L.) Pomel. While root extracts from all the weed species did not induce suicidal germination on *O. crenata* seeds, most of the extracts induced germination of *P. ramosa* except for those obtained from *Amaranthus albus* L., *Amaranthus retroflexus* L. and *Convolvulus arvensis* L. Moderate levels of germination activity were induced in *O. cumana* and *O. minor* seeds by some of the root extracts tested, with strongest induction obtained from *Heliotropium europaeum* L. on *O. cumana* seeds, and from *Silybum marianum* (L.) Gaertn. on *O. minor* seeds. For root extract inhibition of broomrape radicles, the extract from *C. arvensis* roots strongly inhibited radicles of all the broomrape species. While extracts from the other weed species induced low or negligible inhibition of *O. cumana* and *O. crenata* radicle growth, many inhibited *P. ramosa* and *O. minor* radicles. Exceptions were root extracts from *Datura stramonium* L., *Heliotropium europaeum* L., *Malva sylvestris* L., *Solanum nigrum* L. and *Urtica dioica* L., which did not inhibit *P. ramosa* radicles, and those from *A. retroflexus*, *Datura stramonium* L., *Malva sylvestris* L., *Portulaca oleracea* L. and *S. nigrum*, which did not inhibit *O. minor* radicles. Among the active organic extracts assessed, those showing promising chemical profiles were selected for future studies to characterize natural compounds with potential herbicidal activity on early stages of broomrape growth.

**Keywords.** Allelopathy, broomrape weeds, parasitic weed management, sustainable crop protection.

## INTRODUCTION

Among all pests, weeds have the largest economic impact in agriculture (Pimentel *et al.*, 2005), and among weeds, the holoparasitic broomrapes (*Orobanche* and *Phelipanche* species) are particularly noxious, since they compete with crops for nutrients by invading their roots for nutrient extraction. Broomrapes have expanded in importance, becoming threats to food security (Parker, 2009).

For successful crop plant infections, broomrape germination is inhibited until detection of host roots through root-derived germination stimulants (Lechat *et al.*, 2012; Fernández-Aparicio *et al.*, 2009). From each germinated broomrape seed a short radicle emerges that grows towards the host root and then develops a multicellular haustorium. This attaches and penetrates the host root, and forms connections with the host vascular system to withdraw water and nutrients (Rioped and Timko 1995; Joel, 2013). Nutrient withdrawal through haustorial connections results in severe reductions of crop yields (Fernández-Aparicio *et al.*, 2016). Because of synchronization of host-parasite life cycles and the intimate haustorial attachments, application of methods that kill the parasitic weeds without damaging host crop are not easy, so parasitic weeds are amongst the most difficult weeds to control (Fernández-Aparicio *et al.*, 2020).

The most used broomrape control methods are based on systemic herbicides commonly employed for the management other weeds, including inhibitors of aromatic synthesis (glyphosate) or branched-chain amino acid synthesis (imidazolinones and sulfonylureas) (Eizenberg *et al.*, 2013). However, sustainability of herbicide control methods is threatened by the high capacity of weeds to develop resistance coupled with decline in authorized herbicides and lack of discovery of new herbicide mechanisms of action (Duke, 2012; Westwood *et al.*, 2018; Heap, 2021). The mechanisms that broomrapes use to infect crops, i.e. host-induced germination and radicle growth towards host roots for haustorium attachment, can be targeted by natural products which stimulate suicidal broomrape germination in the absence of host plants (Zwanenburg *et al.*, 2016), or which inhibit broomrape radicle growth towards host roots (Cimmino *et al.*, 2014). Development of control of parasitic plants based on natural substances could increase sustainability and efficacy of weed management, and potentially reduce environmental impacts and human health effects.

Allelochemicals are biologically active compounds released from donor plants with allelopathic potential against target plants. The International Allelopathy Society defined allelopathy as any process involving sec-

ondary metabolites produced by plants, algae, bacteria or fungi that influence the growth and development of agricultural and biological systems (International Allelopathy Society, 1996). Many natural compounds have potential as novel agrochemicals (Dayan and Duke, 2014), but only a few microbial or plant metabolites have been screened for their herbicidal activity (Westwood *et al.*, 2018). From screening of microbial or plant toxins, allelochemicals with activity against parasitic weeds have been discovered, which act on seed germination and radicle development (Evidente *et al.*, 2009; Vurro *et al.*, 2009; Evidente *et al.*, 2010; 2011; Cimmino *et al.*, 2014; Cimmino *et al.*, 2015).

The Lithica poem (circa 400 B.C.) noted that “All the pests that out of earth arise, the earth itself the antidote supplies” (Ibn *et al.*, 1781). This inspired the present study to consider the use of root extracts from autotrophic weeds as sources of metabolites in crop rhizosphere to control parasitic weeds. This paper reports the allelopathic activity of root extracts from 17 dicotyledonous weed species that usually thrive on broomrape-infested fields in the south of Spain. Extract activity was assessed for effects on germination and radicle growth of *Orobanche crenata* Forsk., *Orobanche cumana* Wallr., *Orobanche minor* Sm., and *Phelipanche ramosa* (L.) Pomel, which are four of the most noxious broomrape weeds affecting crops in many parts of the world (Parker, 2013).

## MATERIALS AND METHODS

### *Plant material and growth conditions*

Seeds of seventeen weed species (Table 1) were collected during the season of 2016–2017 from a buckwheat field at the Institute for Sustainable Agriculture (IAS-CSIC), Alameda del Obispo Research Center (Córdoba, southern Spain, coordinates 37.856 N, 4.806 W, datum WGS84). The weed species collected frequently occur in southern Spain agricultural fields where *O. crenata* and *O. cumana* also thrive in. In spring 2020, the collected weed seeds were surface sterilized by immersion in 0.5% (w/v) NaOCl and 0.02% (v/v) Tween 20 for 5 min, rinsed thoroughly with sterile distilled water, and dried in a laminar airflow cabinet. The seeds were then sown into pots each containing 1 L of 1:1 sand and peat, which were maintained in a greenhouse for 40 d with day/night regime of 23/20°C, 16/8 h light/dark.

Because broomrapes infect host crop roots and mostly develop underground, extracts from weed roots were used, aiming to identify potential allelopathic signals acting in plant rhizospheres. For collection of roots,

**Table 1.** Weed species investigated for root allelopathic activity against four broomrape species, and mean yields of organic extracts from the CH<sub>2</sub>Cl<sub>2</sub> extractions from dry roots.

Plant family	Plant species	EPPO Code *	Weight in mg of CH <sub>2</sub> Cl <sub>2</sub> organic extract. (% of root dry weight)
Weed species donor of the allelopathic activity			
<i>Amaranthaceae</i>	<i>Amaranthus albus</i>	EPPO:AMAAL	14.40 (0.23)
<i>Amaranthaceae</i>	<i>Amaranthus retroflexus</i>	EPPO:AMARE	19.93 (0.32)
<i>Asteraceae</i>	<i>Conyza bonariensis</i>	EPPO:ERIBO	23.60 (0.39)
<i>Asteraceae</i>	<i>Silybum marianum</i>	EPPO:SLYMA	16.67 (0.28)
<i>Boraginaceae</i>	<i>Heliotropium europaeum</i>	EPPO:HEOEU	21.24 (0.34)
<i>Brassicaceae</i>	<i>Capsella bursa-pastoris</i>	EPPO:CAPBP	22.13 (0.78)
<i>Brassicaceae</i>	<i>Diplotaxis erucoides</i>	EPPO:DIPER	29.62 (0.49)
<i>Brassicaceae</i>	<i>Diplotaxis virgata</i>	EPPO:DIPVG	41.93 (0.70)
<i>Convolvulaceae</i>	<i>Convolvulus arvensis</i>	EPPO:CONAR	77.42 (1.28)
<i>Malvaceae</i>	<i>Malva sylvestris</i>	EPPO:MALSI	36.24 (0.74)
<i>Papaveraceae</i>	<i>Fumaria officinalis</i>	EPPO:FUMOF	39.02 (1.85)
<i>Polygonaceae</i>	<i>Polygonum aviculare</i>	EPPO:POLAV	29.77 (0.49)
<i>Portulacaceae</i>	<i>Portulaca oleracea</i>	EPPO:POROL	25.53 (0.36)
<i>Solanaceae</i>	<i>Datura stramonium</i>	EPPO:DATST	18.64 (0.31)
<i>Solanaceae</i>	<i>Solanum nigrum</i>	EPPO:SOLNI	30.19 (0.49)
<i>Urticaceae</i>	<i>Urtica dioica</i>	EPPO:URTDI	19.35 (0.32)
<i>Zygophyllales</i>	<i>Tribulus terrestris</i>	EPPO:TRBTE	27.52 (0.46)
Parasitic weed species target of the allelopathic activity			
<i>Orobanchaceae</i>	<i>Orobanche crenata</i>	ORACR	
<i>Orobanchaceae</i>	<i>Orobanche cumana</i>	ORACE	
<i>Orobanchaceae</i>	<i>Orobanche minor</i>	ORAMI	
<i>Orobanchaceae</i>	<i>Phelipanche ramosa</i>	ORARA	

\*EPPO Code. European and Mediterranean Plant Protection Organization. <https://gd.eppo.int>.

weed plants at vegetative stages were removed from the pots, their roots were carefully washed in distilled water, quickly dried with filter paper, and immediately frozen and maintained at -80°C until lyophilization.

Broomrape seeds were collected using a 0.6 mm mesh-size sieve (Filtru) from dry inflorescences of *O. crenata* infecting faba bean plants in Spain, *O. cumana* infecting sunflower plants in Turkey, *O. minor* infecting red clover in France and *P. ramosa* infecting oilseed rape in France, and these seeds were stored dry in the dark at 4°C until used.

#### Extraction of dried roots of weed plants

A general method was used to make extractions from the weed roots. Approximately 6 g of dried root tissue of each weed plant was milled in a Waring blender, and the resulting powder was extracted overnight in MeOH-H<sub>2</sub>O (200 mL, 1:1, v:v) under stirring at room temperature in the dark. The resulting sus-

pension was then centrifuged for 1 h at 7000 r.p.m. at 4°C. The supernatant was then extracted with CH<sub>2</sub>Cl<sub>2</sub> (three × 200 mL). The organic extracts were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated under reduced pressure.

#### Germination and radicle growth bioassays

Allelopathic effects of the organic and the aqueous phase extracts from weed roots (Table 1) were assessed for broomrape germination and radicle growth in independent bioassays that were conducted according to previously described protocols (Fernández-Aparicio *et al.*, 2013; Cimmino *et al.*, 2014). Broomrape seed germination is achieved through a two-step process, including warm stratification conditioning followed by chemical stimulation (Lechat *et al.*, 2012). For detection of stimulatory activity of germination, root extracts were dissolved in water, and water without root extracts was used as control. For the discov-

ery of inhibitory activity of radicle growth, the root extracts were mixed with GR24 and, GR24 without root extracts was used as a control.

Seeds of the four broomrape species were surface sterilized by immersion for 5 min in 0.5% (w/v) NaOCl and 0.02% (v/v) Tween 20, and were then rinsed thoroughly with sterile distilled water, and dried in a laminar air flow cabinet. Approximately 100 seeds of each broomrape species were placed separately on 9 mm diameter glass fiber filter paper disks (GFFP) (Whatman International Ltd.) each moistened with 50  $\mu$ L of sterile distilled water and then placed in incubators at 23°C for 10 d inside Parafilm-sealed Petri dishes, to allow seed conditioning. The GFFP disks containing conditioned broomrape seeds were then each transferred onto a sterile sheet of filter paper to remove the excess of water and transferred to a new 10 cm diameter sterile Petri dish.

For germination induction assays, stock solutions of each root organic extract or root aqueous phase dissolved, respectively, in methanol or sterile distilled water, were mixed with sterile distilled water to equivalent concentrations of 0 (control), 10 or 100  $\mu$ g dry extract per mL of distilled water. For radicle growth assays, the stock solutions of each root organic extract or root aqueous phase were each mixed with GR24 to concentrations of 0 (control), 10 or 100  $\mu$ g extract per mL of  $10^{-6}$  M GR24. Triplicate aliquots of 50  $\mu$ L of each sample of each sample were applied to GFFP discs. Treated seeds were incubated in the dark at 23°C for 7 d and percent germination and radicle growth were determined for each GFFP disc as described by Cimmino *et al.* (2014) and Fernández-Aparicio *et al.* (2013), using a stereoscopic microscope (Leica S9i, Leica Microsystems GmbH). Broomrape seed germination was determined by counting the number of germinated seeds from 100 seeds for each GFFP disc (Fernández-Aparicio *et al.*, 2013). For assessing characteristics of radicle growth, the average of ten randomly selected radicles were examined (Westwood and Foy, 1999). The percentage germination induction from each treatment was then calculated relative to the average germination of control seeds (seeds treated with water), and the percentage of radicle growth inhibition for each treatment was calculated relative to the average radicle growth of control treatments (radicles treated with GR24).

#### TLC and HPLC analysis

Analytical and preparative Thin Layer Chromatography (TLC) of plant extracts were carried out on silica gel plates (Kieselgel 60 or F<sub>254</sub>, respectively 0.25 and 0.5 mm), and on reverse phase plates (Kieselgel 60 RP-18,

F<sub>254</sub>, 0.20 mm) (Merck). Eluents used were: CHCl<sub>3</sub>-iso-PrOH 9:1 v/v for direct-phase TLC and MeCN-H<sub>2</sub>O 6:4 v/v for reverse-phase TLC analysis. Spots were visualized by exposure to UV light (254 nm), or by first spraying with 10% H<sub>2</sub>SO<sub>4</sub> in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. The High Performance Liquid Chromatography (HPLC) system used for the analyses of the plant root extracts was from Hitachi, and consisted of a pump (model 5160) and a spectrophotometric UV detector (model 5410) fixed at 254 nm. Metabolites separation was performed using a Phenomenex Luna C<sub>18</sub> reversed-phase column (15  $\times$  4.6 mm; 5  $\mu$ m particle size), which was eluted at a flow rate of 0.5 mL min<sup>-1</sup>. Two HPLC conditions were used. In the first, the gradient commenced from 50% MeCN – 50% 0.1% HCOOH for 20 min, and then linearly increased from 50% to 90% MeCN in 20 min, and remained at these conditions for 10 min. The phase was then followed by a re-equilibrium to the initial gradient composition in 10 min. In the second, the gradient commenced from 60% MeCN – 40% 0.1% HCOOH for 30 min, and then linearly increased from 60% to 90% MeCN in 20 min, and remained at these conditions for 10 min. The phase was then followed by a re-equilibrium to the initial gradient composition for 10 min.

#### Statistical analyses

Percentage data were approximated to normal distributions and stabilize variances using angular transformations ( $180/\pi \times \arcsine(\sqrt{\%/100})$ ). Significance of the weed species donor of activity, the targeted broomrape species and their interactions on induction of suicidal broomrape germination and inhibition of radicle growth was assessed using two-way ANOVA (SPSS version 21.0; SPSS Inc.). For each broomrape species, the significance of mean differences among donor weed species was evaluated by the least significant difference (LSD) ( $P < 0.05$ ). Null hypotheses were rejected at the level of 0.05.

## RESULTS AND DISCUSSION

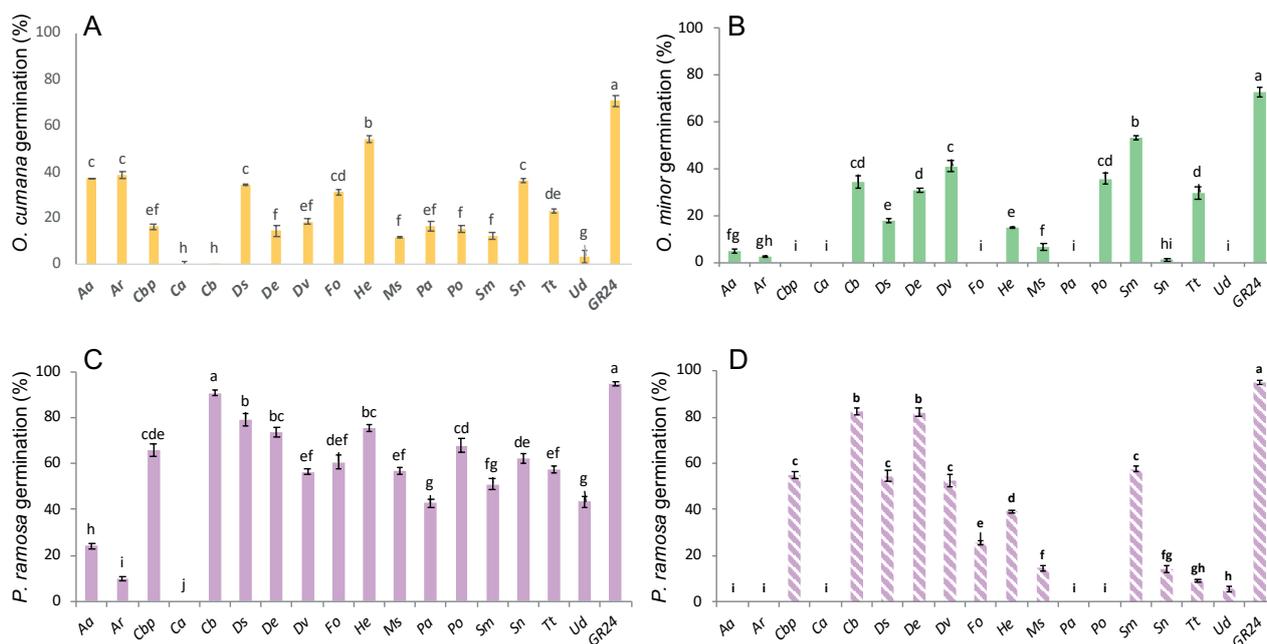
The weed root extractions yielded percentages of organic extract relative to the lyophilized root weight that ranged from 0.23% for *Amaranthus albus* L. to a maximum of 1.85% for *Fumaria officinalis* L. (Table 1). Firstly, the potential was assessed for the weed root extracts to induce broomrape suicidal germination. Germination of broomrape seeds in the absence of host roots is termed “suicidal” because the subsequent

broomrape growth cannot reach host plants which are the sole nutritive sources for the parasitic weeds (Zwanenburg *et al.*, 2016). All of the weed roots extracts were studied at two concentrations (10 or 100 µg of root extract per mL of distilled water) for effects on seeds of the four broomrape species. In all cases, no germination was observed (data not shown) when seeds of the different broomrape species were treated with the negative controls (0 µg of root extract per mL of distilled water). The null germination values induced by the negative controls was expected because broomrape seed germination remains inhibited in absence of germination stimulants (Lechat *et al.*, 2012). The synthetic strigolactone GR24 used as a positive control induced the greatest proportions of mean germination of *O. crenata* seeds,  $71.3 \pm 3.1\%$ , *O. cumana*,  $70.8 \pm 2.5\%$ , *O. minor*,  $72.8 \pm 2.0\%$ , and of *P. ramosa*,  $94.6 \pm 1.1\%$ .

Different protocols have been previously used for isolation of germination stimulants in plant roots. Boutet-Mercey *et al.* (2018) extracted germination stimulants from pea roots, using a method which included 2 h shaking at 4°C of ground frozen roots, and after centrifugation, the supernatant was dissolved in heptane/EtOAc

followed by solid phase extraction. Yoneyama *et al.* (2016) used a different method, which macerated sorghum root pieces in ethanol for up to 3 d. The efficiency of maceration and extraction methods from plant tissues depend on the cellular localization and polarity of the targeted compounds (Halouzka *et al.*, 2020). In the present study, weed root extracts were shown to induce broomrape seed germination (Figure 1). This indicates that extraction method used here was appropriate for identification of broomrape germination stimulants in root tissues.

Germination-inducing activity of weed root extracts was studied for two host-specialist broomrape species *O. crenata* and *O. cumana* and two host-generalist broomrape species *O. minor* and *P. ramosa*. Germination of *O. crenata* seeds was not induced (data not shown) by root extracts from any of the weed species tested at the assessed concentrations (10 or 100 µg dry extract per mL of distilled water). These results indicate that none of the weed root extracts have potential as sources of suicidal germination inducers for *O. crenata*-infested fields. Broomrape seed sensitivity to differing germination stimulants exuded by different crops is used a proxy of host specialization (Fernández-Aparicio *et al.*,



**Figure 1.** Mean seed germination proportions (%) of three broomrape species induced by organic extracts from roots of seventeen weed species, including *Amaranthus albus* L. (Aa); *Amaranthus retroflexus* L. (Ar); Cbp, *Capsella bursa-pastoris* (L.) Medik.; Ca, *Convolvulus arvensis* L.; Cb, *Conyza bonariensis* L.; Ds, *Datura stramonium* L.; De, *Diplotaxis erucoides* (L.) DC.; Dv, *Diplotaxis virgate* (Cav.) DC.; Fo, *Fumaria officinalis* L.; He, *Heliotropium europaeum* L.; Ms, *Malva sylvestris* L.; Pa, *Polygonum aviculare* L.; Po, *Portulaca oleracea* L.; Sm, *Silybum marianum* (L.) Gaertn.; Sn, *Solanum nigrum* L.; Tt, *Tribulus terrestris* Boiss.; Ud, *Urtica dioica* L.. Root extracts were applied at 100 µg (A-C) or 10 µg (D) of root organic extract per mL distilled water to seeds of *Orobanche cumana* Wallr. (A), *Orobanche minor* Sm. (B) or *Phelipanche ramosa* (L.) Pomel. (C and D). For each broomrape species, means accompanied by different letters are significantly different according to the LSD test ( $P < 0.05$ ). Error bars represent the standard error of the mean.

2009; Fernández-Aparicio *et al.*, 2011; Huet *et al.*, 2020). *Orobancha crenata* has a narrow host range mainly specialized to Fabaceae and Apiaceae (Parker, 2013), and consistently with host range, its germination is induced by a narrow range of plant species and molecules when compared with other broomrape species (Fernández-Aparicio *et al.*, 2009).

When weed root extracts were applied to the other three broomrape species at 100 µg dry extract per mL distilled water (Figure 1A, B and C), significant effects were detected in the induction of suicidal germination for broomrape species target of allelopathic activity (ANOVA,  $P < 0.001$ ) and weed species donor of allelopathic activity (ANOVA,  $P < 0.001$ ). The host generalist *P. ramosa* attacks a wide range of crops in Solanaceae, Brassicaceae, Cannabaceae, Fabaceae, Apiaceae, and Asteraceae (Parker, 2013), and its parasitic life cycle is initiated by germination stimulants exuded by roots of many plant species (Fernández-Aparicio *et al.*, 2009). Germination of *P. ramosa* was strongly induced by root extracts of most of the weed species assessed. Exceptions were *A. albus* and *Amaranthus retroflexus* L. which induced low statistically significant germination, and *Convolvulus arvensis* L. that did not induce germination. Consistent with the present results, root exudates of *C. arvensis* were also shown to be inactive inducers of *P. ramosa* seed germination in investigations conducted by Gibot-Leclerc *et al.* (2013). Despite the usual correlations observed in broomrape species between broad host range and high germination sensitivity to different root exudates and germination stimulants released by diverse crop species, these correlations were not observed in the present study for *O. cumana* and *O. minor* against stimulation of different weed root extracts. While the germination of the sunflower specialist *O. cumana* was induced by root extracts of all the plant species assessed except *C. arvensis* and *Conyza bonariensis* L., germination of the host generalist *O. minor* was induced by few weeds species, mainly *C. bonariensis*, *Diplotaxis* spp., *Portulaca oleracea* L., *Silybum marianum* (L.) Gaertn and *Tribulus terrestris* Boiss.

A significant interaction was detected between effects of targeted broomrape species and weed species root extracts (ANOVA,  $P < 0.001$ ). For example, the extracts from *Heliotropium europaeum* L. or *Solanum nigrum* L. gave high activity against seed germination of *O. cumana* and *P. ramosa* but not of *O. minor* or *O. crenata*. In contrast, *C. bonariensis* (Asteraceae) gave high germination induction for *P. ramosa* and *O. minor* but was not active for *O. crenata* or the sunflower specialist *O. cumana*. Root extracts of both species of *Amaranthus* induced high germination of *O. cumana* seeds but gave little or null activity for seeds of *O. crenata*, *O.*

*minor* and *P. ramosa*. The root extract of *C. arvensis* did not induce germination for any of the broomrape species assessed. Different classes of broomrape germination stimulants have been detected in crops, and each crop species exudes a characteristic germination stimulant profile active in specific broomrape species. Specificity in recognition of different classes of germination stimulants by broomrape species has been reported (Fernández-Aparicio *et al.*, 2008; Fernández-Aparicio *et al.*, 2009; Xie *et al.*, 2010; Fernández-Aparicio *et al.*, 2014).

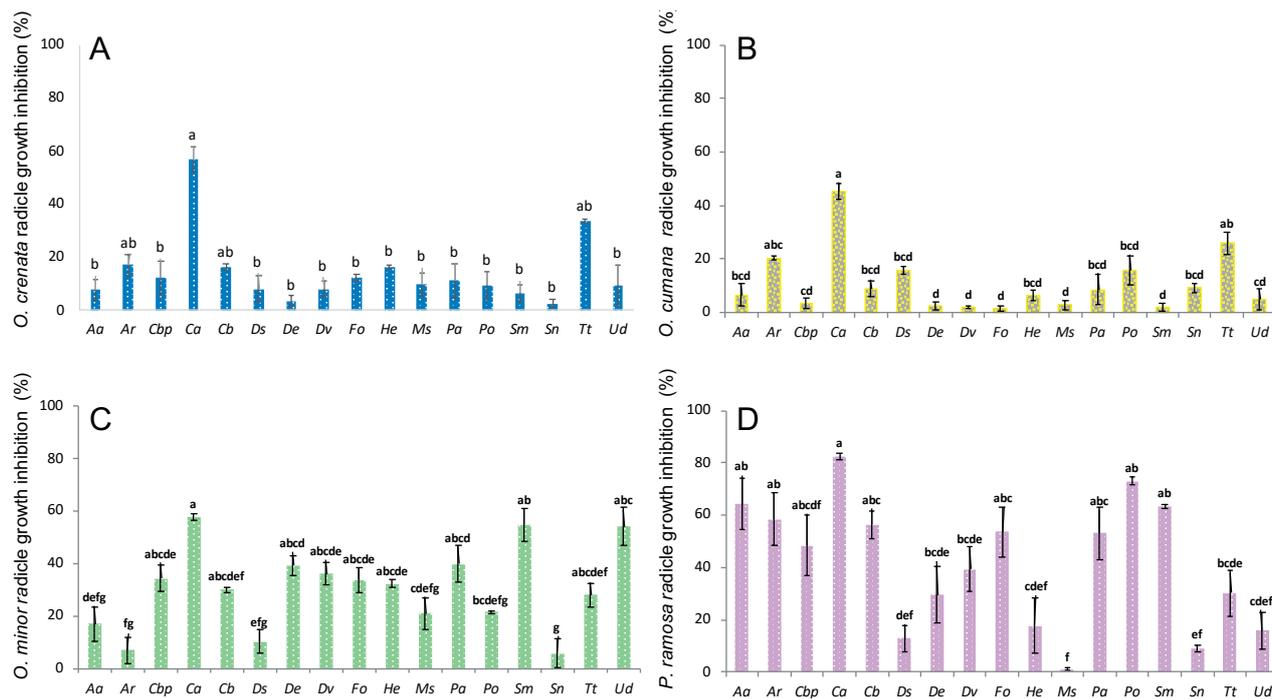
When weed root extracts from most of the assessed weed species were applied to seeds of *O. cumana* and *O. minor* at 10 µg per mL, activity on broomrape germination was reduced to negligible levels (data not shown). The exceptions were the extracts from *S. marianum* on germination of *O. cumana* (mean =  $27.9 \pm 0.4\%$ ) and *O. minor* ( $31.7 \pm 0.9\%$ ), and for germination of *O. cumana* seeds which was induced by extracts from *Diplotaxis virgata* (Cav.) DC. ( $19.9 \pm 0.8\%$ ), *F. officinalis* L. ( $14.9 \pm 1.7\%$ ), *H. europaeum* ( $17.2 \pm 1.0\%$ ), and *Polygonum aviculare* L. ( $9.4 \pm 0.3\%$ ). Seeds of *P. ramosa* were more sensitive to the application of 10 µg per mL extracts (Figure 1D), with the most active extracts were those obtained from *C. bonariensis* ( $82.4 \pm 1.7\%$ ) and *Diplotaxis eruroides* (L.) DC. ( $82.0 \pm 1.7\%$ ). The main classes of identified broomrape germination stimulants are strigolactones (Xie *et al.*, 2010) biosynthetically derived from carotenoids (Matusova *et al.*, 2005). More than 25 strigolactones have been identified in crops (Aliche *et al.*, 2020) and their different structural conformations have species-specific activity for broomrape germination (Fernández-Aparicio *et al.*, 2011). Strigolactones are active on parasitic weed germination at very low concentrations when tested in pure solutions (Xie, 2016; Huet *et al.*, 2020). Other different natural substances (e.g. polyphenols, isothiocyanates, benzonitrils, saponins, and sesquiterpenes) isolated from plants species show broomrape germination stimulation when tested at high concentrations (Cimmino *et al.*, 2018). The unknown metabolites contained in weed root extracts responsible for the suicidal germination activity recorded in the present study gave activity in the complex mixtures of metabolites present in root extracts of each weed investigated. This could indicate significant activity strength, so these compounds will be isolated and characterized in future studies.

The germination inducing activity of aqueous phase root extracts of each weed species was also studied for seeds of *O. crenata*, *O. cumana*, *O. minor*, and *P. ramosa* at 100 or 10 µg of root extract per mL distilled water. In most cases, no germination was observed. The exceptions were when seeds of *P. ramosa* were treated with the aqueous extracts from roots of *D. eruroides* or *D.*

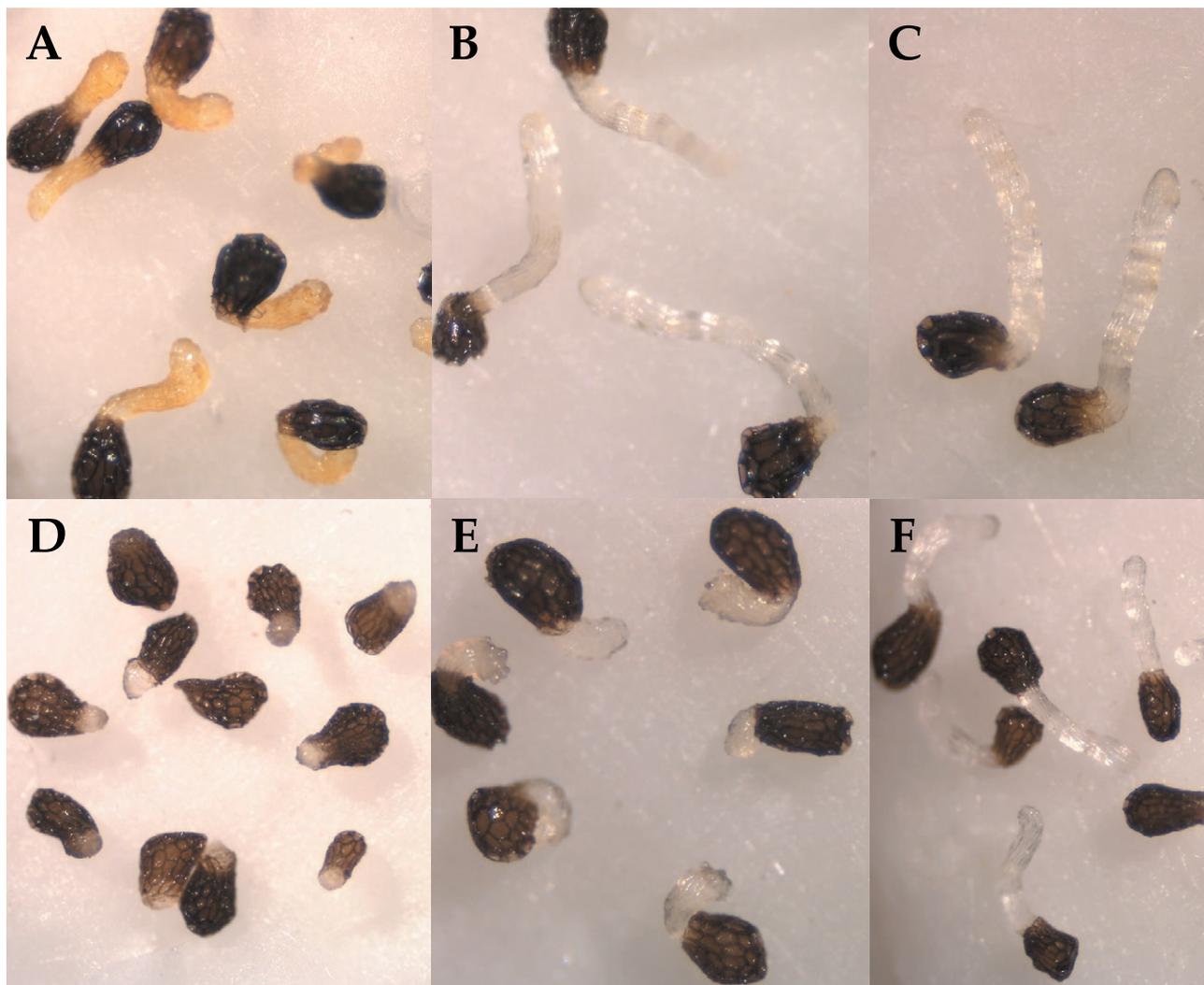
*virgata*. At 100 µg of *D. erucooides* root extract per mL distilled water, mean germination of *P. ramosa* was  $90.2 \pm 0.7\%$ , and for this concentration of extract from *D. virgata* was  $56.8 \pm 2.3\%$ . At 10 µg per mL distilled water, extract from *D. erucooides* gave  $63.6 \pm 0.3\%$  germination of *P. ramosa*, and extract from *D. virgata* gave  $54.6 \pm 2.3\%$  germination of *P. ramosa*. Future characterization of a highly water-soluble molecule with broomrape germination activity could provide a tool for field applications to manage parasitic weeds.

In addition to the identification of potential root extracts as suicidal germination inducers, the organic and aqueous phase extracts from roots of seventeen weed species (Table 1) were also studied for their potential inhibitory activities towards broomrape radicle growth (Figures 2 and 3). All the root extracts were assessed at two concentrations (100 or 10 µg of root extract /mL GR24). No inhibitory activity of radicle growth was observed in any of the aqueous phases regardless the concentration tested nor in the organic extracts applied at the lowest concentration of 10 µg

of root extract /mL GR24 (data not shown). At 100 µg, significant effects on radicle growth were detected for broomrape species target of allelopathic activity (ANOVA,  $P < 0.001$ ) and weed species donors of allelopathic activity (ANOVA,  $P < 0.001$ ) and their interactions (ANOVA,  $P < 0.001$ ). Roots of *C. arvensis* consistently inhibited radicle growth of all the broomrape species studied (Figure 2, Figure 3A and D) in comparison with experimental controls (Figure 3C and F). The mean radicle growth inhibition induced by *C. arvensis* was  $56.9 \pm 4.7\%$  for *O. crenata* radicles,  $45.3 \pm 3.2\%$  for *O. cumana* radicles,  $57.7 \pm 1.1\%$  for *O. minor* radicles, and  $82.5 \pm 1.2\%$  for *P. ramosa* radicles. Besides inhibition of radicle growth, *C. arvensis* root extract induced staining of *O. crenata* radicles as intense yellow with swollen root tips (Figure 3A). The yellow stain was not observed in radicles of any other broomrape species treated with root extract of *C. arvensis* or any other weed. *Orobanche crenata* radicles were not inhibited by root extracts of any other weed species. In contrast, growth of *P. ramosa* radicles was inhibited by most weed extracts, except



**Figure 2.** Mean growth inhibition of broomrape radicle growth induced by organic extracts from roots of seventeen weed species, including: *Amaranthus albus* L. (Aa); *Amaranthus retroflexus* L. (Ar); Cbp, *Capsella bursa-pastoris* (L.) Medik.; Ca, *Convolvulus arvensis* L.; Cb, *Conyza bonariensis* L.; Ds, *Datura stramonium* L.; De, *Diplotaxis erucooides* (L.) DC.; Dv, *Diplotaxis virgate* (Cav.) DC.; Fo, *Fumaria officinalis* L.; He, *Heliotropium europaeum* L.; Ms, *Malva sylvestris* L.; Pa, *Polygonum aviculare* L.; Po, *Portulaca oleracea* L.; Sm, *Silybum marianum* (L.) Gaertn., *Solanum nigrum* L.; Tt, *Tribulus terrestris* Boiss.; Ud, *Urtica dioica* L.. Extracts were assessed at 100 µg per mL of organic extract ( $10^{-6}$ M) on radicles of four broomrape species, including: *Orobanche crenata* Forsk. (A); *Orobanche cumana* Wallr. (B); *Orobanche minor* Sm. (C); and *Phelipanche ramosa* (L.) Pomel (D). Means accompanied by different letters are significantly different according to the LSD test ( $P < 0.05$ ). Error bars represent the standard error of the mean.

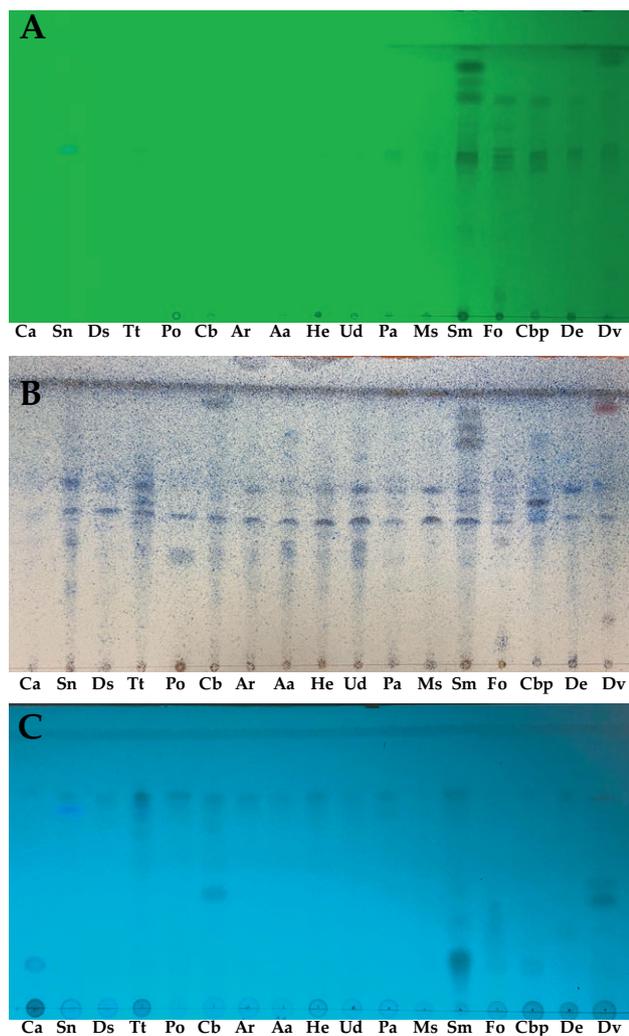


**Figure 3.** Photographs illustrating effects of  $\text{CH}_2\text{Cl}_2$  organic extracts from roots of *Convolvulus arvensis* L. (A and D), and *Portulaca oleracea* L. (B and E), both compared with respective experimental controls (C and F) on radicle growth of (A-C) *Orobanche crenata* Forsk. and (D-F) *Phelipanche ramosa* (L.) Pomel.

those from *Datura stramonium* L., *H. europaeum*, *M. sylvestris*, *S. nigrum* L. and *Urtica dioica* L. Radicle growth of *O. minor* was also inhibited by most of the weed extracts, except those from *A. retroflexus*, *D. stramonium*, *M. sylvestris*, *P. oleracea* and *S. nigrum*. Except for *C. arvensis*, weed extracts had low inhibitory effects on *O. cumana* radicle growth, but low (and statistically significant) inhibitory activity was found for extracts from *A. retroflexus*, *D. stramonium*, *P. oleracea* and *T. terrestris*. The growth inhibition induced by extracts from these four weeds in *O. cumana* was less than 20% of the growth of control radicles.

Organic extracts were first analysed using TLC on direct (Figure 4A and B) and reverse phase (Figure 4C)

plates. Figure 4A and B indicates that all the root extracts gave a similar mixtures of two metabolites at  $R_f = 0.53$  and  $0.63$  when the TLC analysis was performed with  $\text{CHCl}_3$ -*iso*- $\text{PrOH}$  9:1 v/v but revealed with two different methods (exposure to UV light at 254 nm, Figure 4A; and by spraying first with 10%  $\text{H}_2\text{SO}_4$  in  $\text{MeOH}$ , and then with 5% phosphomolybdic acid in  $\text{EtOH}$ , Figure 4B). However, other metabolites were present in the organic extracts from *C. arvensis*, *S. nigrum*, *T. terrestris*, *S. marianum*, *P. oleracea*, *F. officinalis*, *C. bursa-pastoris* and *D. virgata*. These results were also confirmed by the chromatographic profiles obtained using reverse phase plates eluted with  $\text{MeCN-H}_2\text{O}$  6:4 v/v and spots visualized by exposure to UV light (254 nm) (Figure 4C).



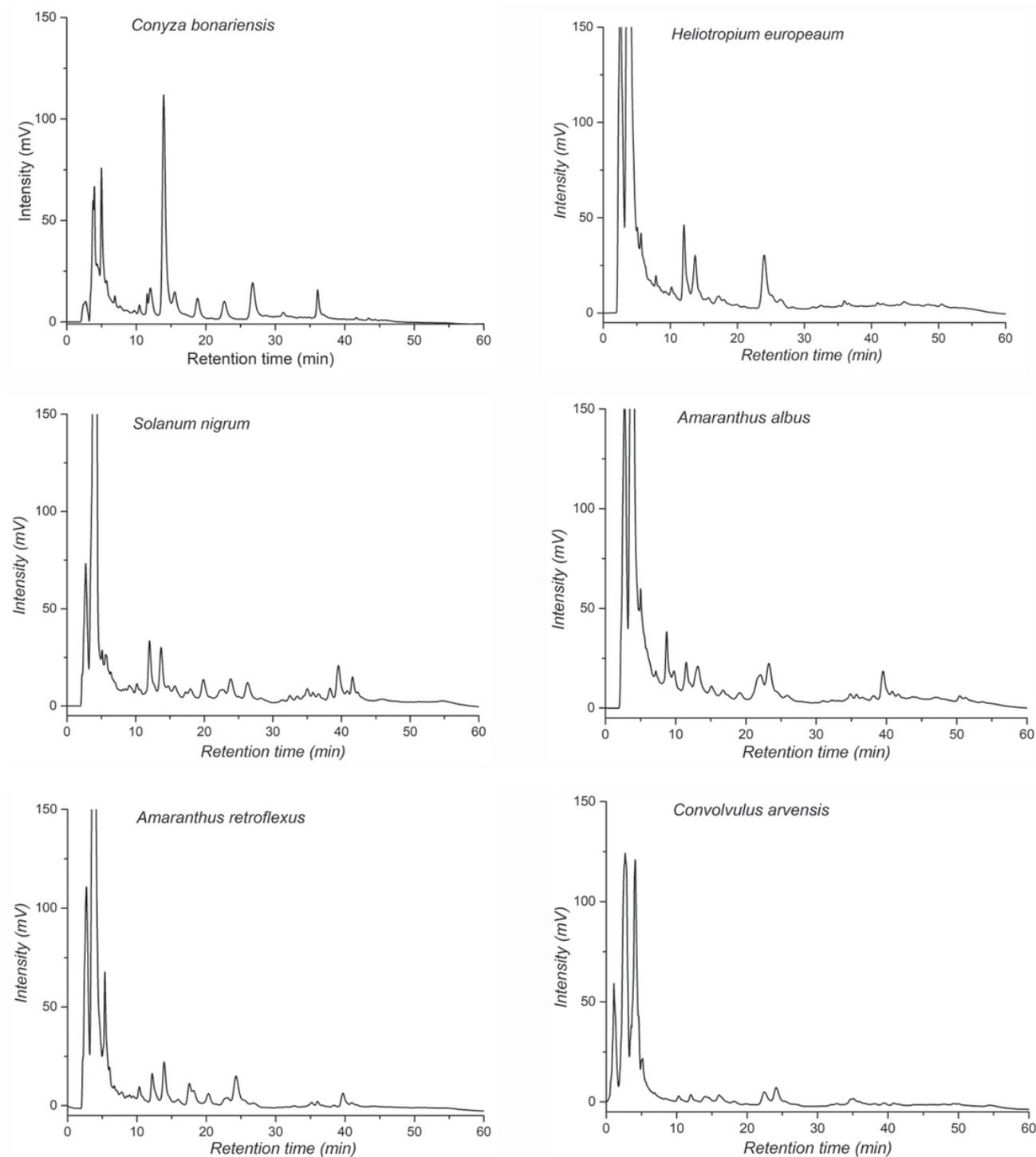
**Figure 4.** TLC analyses of plant root extracts from: Ca, *Convolvulus arvensis* L.; Sn, *Solanum nigrum* L.; Ds, *Datura stramonium* L.; Tt, *Tribulus terrestris* Boiss.; Po, *Portulaca oleracea* L.; Cb, *Conyza bonariensis* L.; Ar, *Amaranthus retroflexus* L.; Aa, *Amaranthus albus* L.; He, *Heliotropium europaeum* L.; Ud, *Urtica dioica* L.; Pa, *Polygonum aviculare* L.; Ms, *Malva sylvestris* L.; Sm, *Silybum marianum* (L.) Gaertn.; Fo, *Fumaria officinalis* L.; *Capsella bursa-pastoris* (L.) Medik.; De, *D. eruroides* (L.) DC. and Dv, *D. virgate* (Cav.) DC.. A, Chromatograms obtained using silica gel plates eluted with  $\text{CHCl}_3$ -iso-PrOH 9:1 v/v and spots visualized by exposure to UV light (254 nm); B, Chromatograms obtained using silica gel plates eluted with  $\text{CHCl}_3$ -iso-PrOH 9:1 v/v and visualized by spraying first with 10%  $\text{H}_2\text{SO}_4$  in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min; C, Chromatogram obtained using reverse phase plates eluted with MeCN- $\text{H}_2\text{O}$  6:4 v/v and spots visualized by exposure to UV light (254 nm).

The organic extracts were also analyzed using HPLC, using the conditions designated a (see above). The optimized HPLC analyses (using the conditions designated b, above) of extracts from the active plants are reported in

Figure 5. In particular, the extracts analyzed were those of *C. bonariensis*, which gave high germination inducing activity for *P. ramosa* and *O. minor* but were not active for *O. cumana* or *O. crenata*; *H. europaeum* and *S. nigrum* which gave high seed germination induction for *O. cumana* and *P. ramosa*, but not for *O. minor* or *O. crenata*; *A. albus* and *A. retroflexus* which gave high levels of germination of *O. cumana* seeds, but low or no activity for seeds of *O. crenata*, *O. minor* or *P. ramosa* and root extracts of *C. arvensis* which did not induce germination in any of the broomrape species assessed.

Among these active organic extracts, the present study has identified those obtained from *C. arvensis*, *C. bonariensis*, *P. oleracea*, and *D. euricoides* for future isolation and characterization of bioactive metabolites with herbicidal activity against early stages of broomrape development, particularly focusing on the promising TLC profiles and HPLC analyses. Phytochemical analysis of these weed species components have been reported for some of the plants assessed in the present study. From *C. arvensis*, carbohydrates, coumarins, saponins, flavanoids, lipids, steroids, terpenoids, alkaloids, tannins and lactones have been isolated from *Convolvulus* (Salehi *et al.*, 2020), while homoisoflavonoids, terpenoids, alkaloids, cerebrosides, coumarins, flavonoids and organic acids have been isolated from *P. oleracea* (Yan *et al.*, 2012; Wang *et al.*, 2017). Phenylpropanoids, flavonoids, sesquiterpenes, alkaloids, and phenolic acids have been isolated from *A. retroflexus* (Fiorito *et al.*, 2017), and glycosides and monoterpenes have been isolated from *C. bonariensis* (Kong *et al.*, 2001; Zahoor *et al.*, 2010; Miranda *et al.*, 2015). Flavonoids and their glycosides have been obtained from *D. eurocoides* (Salah *et al.*, 2015; Hussein *et al.*, 2017). Roots extract from these plants could be used combined with other broomrape management methods (e.g. crop resistance to haustorial infection, modification of crop sowing dates, fertilizer applications, biological control agents) as components of strategies for integrated broomrape management. These strategies will reduce the use of synthetic herbicide for eco-friendly weed control practices (Scavo and Mauromicale, 2020).

In conclusion, this paper is the first report of the allelopathic potential of seventeen weeds for management of *Orobanche* and *Phelipanche* species which can be responsible of heavy losses in important crops. Assessments of weed root organic extracts were carried out to characterize antagonist activity as well as corresponding chemical composition. These results are an important starting point for the isolation and characterization of metabolites with allelopathic activity against parasitic weeds. Further investigations will potentially



**Figure 5.** Optimized HPLC analysis of the extracts of the active plants: *Conyza bonariensis* L., *Heliotropium europaeum* L., *Solanum nigrum* L., *Amaranthus albus* L., *Amaranthus retroflexus* L. and *Convolvulus arvensis* L.. The HPLCs were obtained using a 0.5 mL min<sup>-1</sup> flow rate and the gradient: commencing from 60% MeCN - 40% 0.1% HCOOH for 30 min, and then linearly increasing from 60% to 90% MeCN in 20 min, and remaining at these conditions for 10 min. The phase was then followed by a re-equilibrium to the initial gradient composition for 10 min.

allow development of broomrape biocontrol method based on formulations containing active metabolites.

#### ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministry of Science and Innovation (RYC-2015-18961 and AGL2017-87693-R) and from a CSIC-ALGOSUR research contract. Antonio Evidente is associated with the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy. Authors wish to thank INPS (Istituto Nazionale Previdenza Sociale) for funding a Ph.D. grant to Gabriele Soriano.

#### LITERATURE CITED

- Aliche E.B., Screpanti C., De Mesmaeker A., Munnik T., Bouwmeester H.J., 2020. Science and application of strigolactones. *New Phytologist* 227: 1001–1011.
- Boutet-Mercey S., Perreau F., Roux A., Clave G., Pillot J.P., ... Boyer F.D., 2018. Validated method for strigolactone quantification by ultra high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry using novel deuterium labelled standards. *Phytochemical Analysis* 29: 59–68.
- Cimmino A., Fernández-Aparicio M., Andolfi A., Basso S., Rubiales D., Evidente, A., 2014. Effect of fungal and plant metabolites on broomrapes (*Orobanche* and *Phelipanche* spp.) seed germination and radicle growth. *Journal of Agricultural and Food Chemistry* 62: 10485–10492.
- Cimmino A., Masi M., Evidente M., Superchi S., Evidente A., 2015. Fungal phytotoxins with potential herbicidal activity: chemical and biological characterization. *Natural Product Reports* 32: 1629–1653.
- Cimmino A., Masi M., Rubiales D., Evidente A., Fernández-Aparicio M., 2018. Allelopathy for parasitic plant management. *Natural Product Communications* 13: 289–294.
- Dayan F.E., Duke S.O., 2014. Natural compounds as next-generation herbicides. *Plant Physiology* 166: 1090–1105.
- Duke S.O., 2012. Why have no new herbicide modes of action appeared in recent years? *Pest Management Science* 68: 505–512.
- Eizenberg H., Hershenhorn J., Ephrath J.H., Kanampiu F., 2013. Chemical control. In *Parasitic Orobanchaceae* (Joel D.M., Gressel J., Musselman L.J., Eds.). Springer: Berlin/Heidelberg, Germany, pp. 415–432.
- Evidente A., Cimmino A., Fernández-Aparicio M., Andolfi A., Rubiales D., Motta A., 2010. Polyphenols, including the new peapolyphenols A–C, from pea root exudates stimulate *Orobanche foetida* seed germination. *Journal of Agricultural and Food Chemistry* 58: 2902–2907.
- Evidente A., Cimmino A., Fernández-Aparicio M., Rubiales D., Andolfi A., Melck D., 2011. Soyasapogenol B and trans-22-dehydrocampesterol from common vetch (*Vicia sativa* L.) root exudates stimulate broomrape seed germination. *Pest Management Science* 67: 1015–1022.
- Evidente A., Fernández-Aparicio M., Cimmino A., Rubiales D., Andolfi A., Motta A., 2009. Peagol and peagoldione, two new strigolactone like metabolites isolated from pea root exudates. *Tetrahedron Letters* 50: 6955–6958.
- Fernández-Aparicio M., Andolfi A., Evidente A., Pérez-de-Luque A., Rubiales D., 2008. Fenugreek root exudates show species-specific stimulation of *Orobanche* seed germination. *Weed Research* 48: 163–168.
- Fernández-Aparicio M., Cimmino A., Evidente A., Rubiales D., 2013. Inhibition of *Orobanche crenata* seed germination and radicle growth by allelochemicals identified in cereals. *Journal of Agricultural and Food Chemistry* 61: 9797–9803.
- Fernández-Aparicio M., Delavault P., Timko M., 2020. Management of infection by parasitic weeds: A review. *Plants* 9: 1184.
- Fernández-Aparicio M., Flores, F., Rubiales, D., 2009. Recognition of root exudates by seeds of broomrape (*Orobanche* and *Phelipanche*) species. *Ann. Bot.* 103: 423–431.
- Fernández-Aparicio M., Kisugi T., Xie X., Rubiales D., Yoneyama K., 2014. Low strigolactone root exudation: A novel mechanism of broomrape (*Orobanche* and *Phelipanche* spp.) resistance available for faba bean breeding. *Journal of Agricultural and Food Chemistry* 62: 7063–7071.
- Fernández-Aparicio M., Yoneyama K., Rubiales D., 2011. The role of strigolactones in host specificity of *Orobanche* and *Phelipanche* seed germination. *Seed Science Research* 21: 55–61.
- Fernández-Aparicio, M., Flores F., Rubiales D., 2016. The effect of *Orobanche crenata* infection severity in faba bean, field pea and grass pea productivity. *Frontiers in Plant Science* 7: 1049.
- Fiorito S., Epifano F., Palmisano R., Genovese S., Taddeo V.A., 2017. A re-investigation of the phytochemical composition of the edible herb *Amaranthus retroflexus* L. *Journal of pharmaceutical and biomedical analysis* 143: 183–187.
- Gibot-Leclerc S., Abdennebi-Abdemessed N., Reibel C., Colbach N., 2013. Non-host facilitators, a new category that unexpectedly favours parasitic weeds. *Agronomy for Sustainable Development* 33: 787–793.

- Halouzka R., Zeljkovic S.C., Klejdus B., Tarkowski P., 2020. Analytical methods in strigolactone research. *Plant Methods* 16: 76.
- Heap I. *The International Survey of Herbicide Resistant Weeds*. www.weedscience.com. Accessed: March 21, 2021.
- Huet S., Pouvreau J.-B., Delage E., Delgrange S., Marais C., ... Poulin L., 2020. Populations of the parasitic plant *Phelipanche ramosa* influence their seed microbiota. *Frontiers in Plant Science* 11: 1075.
- Hussein S.R., Marzouk M.M., Kassem M.E., Abdel Latif R.R., Mohammed R.S., 2017. Chemosystematic significance of flavonoids isolated from *Diplo-taxis acris* (Brassicaceae) and related taxa. *Natural Product Research* 31: 347–350.
- Ibn H., Tyrwhitt T., 1781. Orpheus Peri Lithôn de Lapidibus, Poema Orpheo a Quibusdam Adscriptum. Payne, White, and Elmsly, London.
- International Allelopathy Society (IAS) Constitution and Bylaws 1996. [Online].
- Joel D.M., 2013. Functional structure of the mature haustorium. In *Parasitic Orobanchaceae*; (D.M. Joel, J. Gressel, L.J. Musselman ed.) Springer, Berlin/Heidelberg, Germany, 25–60.
- Kong L.D., Abliz Z., Zhou C.X., Li L.J., Cheng C.H.K., Tan R.X., 2001. Glycosides and xanthine oxidase inhibitors from *Conyza bonariensis*. *Phytochemistry* 58: 645–651.
- Lechat M.M., Pouvreau J.B., Péron, T., Gauthier M., Montiel G., ... Delavault P. 2012. PrCYP707A1, an ABA catabolic gene, is a key component of *Phelipanche ramosa* seed germination in response to the strigolactone analogue GR24. *Journal of Experimental Botany* 63: 5311–5322.
- Matusova R., Rani K., Verstappen F.W.A., Franssen M.C.R., Beale M.H., Bouwmeester, H.J., 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. *Plant Physiologist* 139: 920–934.
- Miranda C.A.S.F., Carvalho M.L.M., Gomes M.S., Santiago J.A., Santiago W.D.; Teixeira M.L., 2015. Evaluation of the chemical composition and allelopathic potential of essential oils from three species of *Asteraceae* against seed germination and seedling vigor of lettuce *Journal of Advanced Pharmaceutical Technology & Research* 11: 3669–3677.
- Parker C., 2009. Observations on the current status of *Orobanche* and *Striga* problems worldwide. *Pest Management Science* 65: 453–459.
- Parker C., 2013. The parasitic weeds of the Orobanchaceae. In *Parasitic Orobanchaceae*. Joel D.M., Gressel J., Musselman L.J., Eds.; Springer: Berlin/Heidelberg, Germany; pp. 313–344.
- Pimentel D., Zuniga R., Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52: 273–288.
- Riopel J.L., Timko M.P., 1995. Haustorial initiation and differentiation. In: *Parasitic Plants*. (M.C. Press, J.D. Graves, ed.) Chapman & Hall, London, UK, 39–79.
- Salah N.B., Casabianca H., Jannet H.B., Chenavas S., Sanglar C., Fildier A., Bouzouita N., 2015. Phytochemical and biological investigation of two *Diplo-taxis* species growing in Tunisia: *D. virgata* & *D. erucoides*. *Molecules* 20: 18128–18143.
- Salehi B., Krochmal-Marczak B., Skiba D., Patra J.K., Das S.K., Das G., Martorell M., 2020. *Convolvulus* plant-A comprehensive review from phytochemical composition to pharmacy. *Phytotherapy Research* 34: 315–328.
- Scavo, A., Mauromicale, G., 2020. Integrated Weed Management in Herbaceous Field Crops. *Agronomy* 10(4): 466. <https://doi.org/10.3390/agronomy10040466>
- Vurro M., Boari A., Evidente A., Andolfi A., Zermene N., 2009. Natural metabolites for parasitic weed management. *Pest Management Science* 65: 566–571.
- Wang H., Zhang L., Wang Y., 2017. Isolating and identifying organic acids from *Portulaca oleracea* and determining their anti-cyanobacterial activity. *Polish Journal of Environmental Studies* 26: 441–445.
- Westwood J.H., Charudattan R., Duke S.O., Fennimore S.A., Marrone P., ... Swanton C., Zollinger R., 2018. Weed Management in 2050: Perspectives on the Future of Weed Science. *Weed Science*, 10.1017/wsc.2017.78.
- Westwood, J.H., Foy, C.L. 1999. Influence of nitrogen on germination and early development of broomrape (*Orobanche* spp.). *Weed Science* 47: 2–7.
- Xie X., 2016. Structural diversity of strigolactones and their distribution in the plant kingdom. *Journal of Pesticide Science* 41: 175–180.
- Xie X., Yoneyama K., Yoneyama K., 2010. The strigolactone story. *Annual Review of Phytopathology* 48: 93–117.
- Yan J., Sun L.R., Zhou Z.Y., Chen Y.C., Zhang W.M., Dai H.F., Tan J.W., 2012. Homoisoflavonoids from the medicinal plant *Portulaca oleracea*. *Phytochemistry* 80: 37–41.
- Yoneyama K., Xie X., Nomura T., Yoneyama K., 2016. Extraction and measurement of strigolactones in sorghum roots. *Bio-protocol* 6: e1763.
- Zahoor A., Siddiqui I.N., Khan A., Ahmad V.U., Ahmed A., Hassan Z., Iqbal, S. 2010. Two new glycosides from *Conyza bonariensis*. *Natural Product Communications* 5: 1099–1102.
- Zwanenburg B., Mwakaboko A.S., Kannan C., 2016. Sui-cidal germination for parasitic weed control. *Pest Management Science* 72: 2016–2025.