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Plant extracts to manage the parasitic weed branched broomrape (*Phelipanche ramosa*)

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Summary. Some weeds have parasitic lifestyles, causing severe problems in agriculture. These plants include *Phelipanche ramosa* (L.) Pomel (branched broomrape). Greenhouse and nursery trials were carried out to assess control of *P. ramosa* using organic extracts from 14 plant species. The parameters recorded were counts of living and dead tubercles of *P. ramosa* and fresh weights of living tubercles. Organic extract of *Olea europea* reduced lengths of germ tubes during *P. ramosa* seed germination, and extracts of *Bidens bipinnata* and *Dittrichia viscosa* reduced production and development of the parasite's tubercles, with very encouraging results in reducing seed germination rates. This research provides knowledge insights on the potential use of plant secondary metabolites to limit spread of *P. ramosa*, addressing an increasing challenge for organic crop production.

Keywords. Parasitic weeds, organic farming, allelopathy, agricultural sustainability.

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INTRODUCTION

Root parasitic weeds pose serious problems in agriculture, causing severe crop losses in yield and quality. These weeds rely on neighbouring host plants for their whole life cycles, depleting water and nutrients, and severely reducing host plant growth. Their life cycles are spent mostly underground, and when they emerge, most of the damage they cause has already been accomplished. These parasitic weeds also produce large numbers of long-lived seeds that determine the formation of persistent seedbanks, making the soil infestation, after occurring, almost impossible to remove (Fernández-Aparicio *et al.*, 2020).

The most damaging root parasitic weeds, including broomrapes (*Orobanche* and *Phelipanche* spp.) and witchweeds (*Striga* spp.), are widespread in Europe, Africa, and Asia, while other parasitic weeds (*Alec-*

tra, *Aeginetia*, *Buchnera*, and *Rhamphicarpa*) are also becoming increasingly important. Due to their characteristics, traditional control strategies based on herbicides, agronomic practices, and resistant varieties, are only partially effective, particularly in low-input crops (Parker, 2021; Rubiales, 2023), or cannot be fully applied in organic farming and other agriculture systems. To effectively manage parasitic weeds, it is important to understand the physiological and molecular mechanisms of their germination, haustorium development, as well as crop resistance, and to discover new herbicides and bioherbicides and their applications for control of parasitic weeds (Kebede and Ayana, 2018).

Phelipanche ramosa (L.) Pomel., commonly known as branched broomrape, has a broad host range, primarily including *Solanaceae* hosts such as tomato, potato, and tobacco. This parasitic weed also infects other plant including *Brassicaceae*, *Cannabaceae*, *Fabaceae*, *Apiaceae*, and *Asteraceae* (Musselman and Parker, 1982). *P. ramosa* is native in Europe, Africa, and Asia, and has high adaptability and evolution to expand presence and host range. Furthermore, with the high dispersal capabilities, new areas are being infested by *P. ramosa* (Rubiales, 2020). The detrimental impacts of this parasitic weed on host plants surpasses the anticipated consequences based on the parasite's dry weight. This could be due to arid conditions or imbalanced effects on the fruiting capacity of host plants compared to their vegetative biomass. Documented yield losses due to broomrape in tomato and tobacco crops are likely to range from 30% to 50% (Parker, 2013).

The present study aimed to assess the potential of plant extracts as natural herbicides against *P. ramosa*. The focus was to assess their effects during the most vulnerable stages of *P. ramosa* growth cycle: seed germination, tubule elongation, haustorium attachment, and tubercle development. By targeting these stages, the aim was to develop an effective and precise strategy for controlling *P. ramosa*. This approach utilizes the unique properties of selected plant compounds to disrupt specific growth processes, offering an avenue for eco-friendly and sustainable weed management. The plants assessed in this study are from families known for producing secondary metabolites or phytotoxic substances, enhancing the likelihood of identifying potent natural herbicides. Additionally, the selected plants are prevalent throughout the Mediterranean region, and are easily propagated and multiplied, providing practical advantages for further experimentation and application of their metabolites. Except for *Dittrichia viscosa* (L.) Greuter, these plant species assessed here have not been previously assessed against *P. ramosa*. This study was a preliminary screening of the plant species with potential bioactive organic compounds, and the aim was to find novel extracts, to purify them, and obtain compounds with potential as broomrapes bioherbicides.

MATERIALS AND METHODS

Extraction

Plant materials (leaves and green twigs) were harvested during November 2022 to March 2023 from 14 plant species. These included; *Ailanthus altissima* (Mill.) Swingle, *Amaranthus retroflexus* (L.), *Avena fatua* (L.), *Bidens bipinnata* (L.), *Brassica nigra* (L.), *Cirsium arvense* (L.) Scop., *Diplotaxis erucoides* (L.), *Dittrichia viscosa* (L.) Greuter, *Ecballium elaterium* (L.) A.Rich., *Juglans regia* (L.), *Olea europaea* (L.), *Oxalis pes-caprae* (L.), *Solanum nigrum* (L.), and *Sorghum halepense* (L.). The plants were harvested from the experimental field at the Mediterranean Agronomic Institute of Bari (CIHEAM Bari), Valenzano, southern Italy.

The harvested plant materials were cleaned and dried in a fan oven at 45°C for 3 d. They were then ground until to powder and kept in air-sealed plastic bags at 8°C, until further processing and extraction.

Dried plant material (100 g of each plant) was added to a glass flask, and 500 mL of methanol solution (H_2O: MeOH, 1:1, v:v) was added. The flasks were then kept in stirred conditions at room temperature (25°C) for 24 h, to separate the aqueous extracts (Fernández-Aparicio *et al.*, 2021). The obtained solutions were then filtered from solid plant material using gauze cloth, and centrifuged at 7000 rpm at 5°C for 10 min. Supernatants were separated from the solid phases after centrifugation. Dichloromethane (CH_2Cl_2; 600 mL) was then added (three times, each of 200 mL CH_2Cl_2) as organic solvent to the supernatants, to obtain the organic extracts. Separating funnels were then used to separate the aqueous extracts from organic extracts. The organic extracts were then dehydrated and filtered with cotton wool, and evaporated using a rotavapor (Dor and Hershenhorn, 2012). The concentrated organic extracts were purified from any methanol traces by nitrogen evaporator, and were then kept in freezer at -12°C.

Seed germination test

The aim of this test was to measure effects of the plant extracts on the germination of *P. ramosa*. The test was conducted using the design described by Boari and Vurro (2004), to verify which organic extracts of the

harvested plants had potential to suppress or inhibit germination of *P. ramosa* seeds. After selecting and collecting the 14 adventitious plants, the 14 organic extracts were compared with experimental controls containing water, GR_{24}[1] and MeOH. For each individual test, a triangular piece of filter paper was placed into a Petri dish (total of 45 dishes), and 1 mL of water solution containing organic extract (0.5 mg mL^{-1}). Conditioned *P. ramosa* seeds (approx. 100 seeds) were added to each Petri dish, and the dishes were then wrapped in plastic wrap and covered with aluminum foil. The dishes were then placed in the incubator. After 72 h, germination percentages were observed using a binocular microscope. Seeds with emerged radicles were classified as germinated. The design used for this experiment was a completely random block with three replicates. Treatments that reduced *P. ramosa* seed germination were considered effective.

Assessment of infectivity of tomato by Phelipanche ramosa *in plastic file bags*

Tomato seeds (*Solanum lycopersicum* cv. Marmande) were sown into vermiculite in Styrofoam trays and were irrigated frequently as needed. The resulting seedlings were fertilized once each week with Hoagland solution (Chen *et al.*, 1961).

Modified plastic file bags were prepared for this test (Amsellem *et al.*, 2001; Vurro *et al.*, 2006). An A4 plastic files were sealed at the edges with a heat sealer and were each cut in the middle to form a window-like flipper with the same dimensions as a fiberglass sheet held inside the plastic file. A plastic tube (20 cm length) was also inserted into each file. The fiberglass sheet was moistened with 12 mL of sterilized tap water. Seven plastic crates were prepared to host 70 of these plastic file bags assessments of *P. ramosa* infection of tomato seedlings. Dry *P. ramosa* seeds (30 mg) were then sprinkled evenly distributed on the fiberglass sheet. These plastic file bags were then placed inside a plastic crate modified for the purpose. The experimental design was a completely randomized design with ten replicates. One day later, tomato seedlings (24 d old, two to three true leaves) were removed from the trays. Their roots were washed and cleaned from vermiculite and the plants were inserted into each plastic bag, positioned on the fiberglass paper, and fixed in place by scotch tape. These plants were irrigated and fertilized with Hoagland solution periodically. Organic extracts (500 mL each; 0.5 mg mL^{-1}) were prepared from *A. altissima*, *B. bipinnata*, *C. arvense*, *D. viscosa*, *J. regia*, and *O. europaea*, so that each tomato plant received 10 mL of treatment solution. The treatments were applied 26 d after the plants were transplanted. The trial lasted about 2 months.

At the end of the trial, living and dead tubercles were counted, and fresh weights of living tubercles were measured. The tubercles were divided into three categories, A (small), B (medium), or C (large), based on their surface areas (visible from outside the bag) as A, <0.3 cm^2, B, 0.3–0.6 cm^2, or C, >0.6 cm^2. Measurement of surface areas and counting of the tubercles were done using ImageJ version 1.54d, free software for image analysis (Schneider *et al.*, 2012).

Pot experiment

This experiment aimed to evaluate effectiveness of treatments under greenhouse and nursery conditions. Tomato seeds (cv. Marmande) were sowed into seedling trays containing peatmoss. The trays were kept in a growth chamber under optimal conditions and irrigated as needed. Seventy pots (each 1 L capacity) were prepared, each containing a layer of tissue paper at the bottom to block the drainage holes and prevent soil escape. A layer (2–3 cm thick) of peatmoss mixed with soil was added, followed by a layer of soil inoculated with *P. ramosa* seeds (approx. 0.85 L of soil mixed with 24 mg of *P. ramosa* seeds). Tomato plants (23 d old, 3 to 4 true leaves) were then transplanted into the pots, and another 2–3 cm layer of peatmoss was added to each pot. The pots were irrigated periodically from above with water, and Hoagland solution was added for fertilization. The experiment was a completely randomized design with ten replicates.

Each pot received 50 mL (conc. 0.5 mg mL^{-1}) of extracts from either *A. altissima*, *B. bipinnata*, *C. arvense*, *D. viscosa*, *J. regia*, and *O. europaea*. Final evaluations were carried out 20 d after treatment. The trial lasted 2 months. At the end of the trial, numbers of living *P. ramosa* tubercles were counted, and fresh weights of tubercles were measured. The tubercles were divided into three categories, as A (small, <0.3 cm^2), B (medium, 0.3–0.6 cm^2), or C (large, >0.6 cm^2).

Statistical analyses

RStudio IDE of the R programming language (RStudio Team, 2020) was used to analyze the results obtained and determine statistical significance of mean differences, using core packages (R Core Team, 2023),

[1] A synthetic germination stimulant for broomrape seeds (Lechat *et al.*, 2012).

as well as the "tidyverse", "knitr", "car", "haven", "TukeyC", "ggpubr", "dunn.test", "reshape2", "superb", and "ggplot2" metapackage (John Fox and Sanford Weisberg, 2019; Wickham *et al*., 2019; Jose *et al*., 2023; Smith, 2023; Yihui Xie, 2023).All the variables were first tested for normal distributions using the Shapiro-Wilk normality test, and variables that followed normal distributions ($P > 0.05$) were subjected to Analysis of Variance (2-way ANOVA) tests, followed by Tukey's HSD post-hoc tests at $P = 0.05$. Means were then visualized using boxplots. Variables that did not follow normal distributions (Shapiro-Wilk; $P > 0.05$) were analyzed using analysis of variance (Kruskal–Wallis, non-parametric) of independent samples, followed by Dunn's post-hoc test at $P = 0.05$, then visualization using Boxplots.

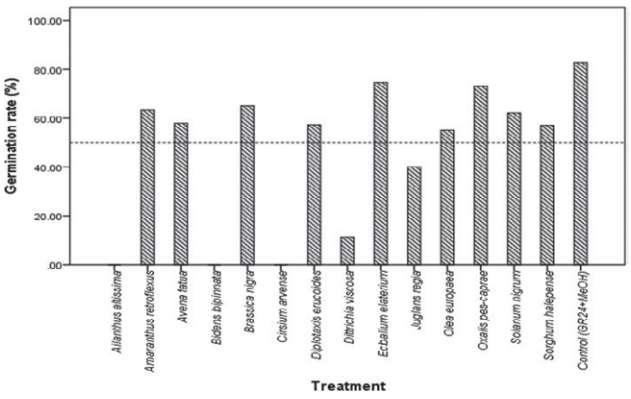

Figure 1. Germination (%) of preconditioned *Phelipanche ramosa* seeds treated with different plant extracts (0.5 mg mL^{-1}), and synthetic germination stimulant (Control; see materials and methods for the details of the bioassay).

RESULTS

Seed germination assays

The organic extracts of three plants, *A. altissima*, *B. bipinnata*, and *C. arvense*, applied at 0.5 mg mL^{-1} to preconditioned seeds of *P. ramosa* caused complete inhibition of germination. (from 81% to 0%); the extract of *D. viscosa* was also active, reducing germination rate by 68.5% (from 81% to 12.5%), and the extract from *J. regia* gave germination of 44% (Figure 1). All the other extracts reduced *P. ramosa* seed germination to a lesser extent in comparison with the control. The extracts which reduced germination to less than 50% were selected for further assessments in plastic bag bioassays (below) for the *P. ramosa* infection phase. The extracts from *A. altissima*, *B. bipinnata*, *C. arvense*, *D. viscosa* and *J. regia* were selected for the tomato plant assay (below), together with that from *O. europaea*. that, although reduced germination by only 26% (from 81 to 55%), induced an unusual deformation to the germ tube of the *P. ramosa* seed (Figure 2).

Tomato infectivity test in plastic file bags

This test aimed to evaluate effects of the selected organic extracts applied to tomato plants during the *P. ramosa* infection phase.

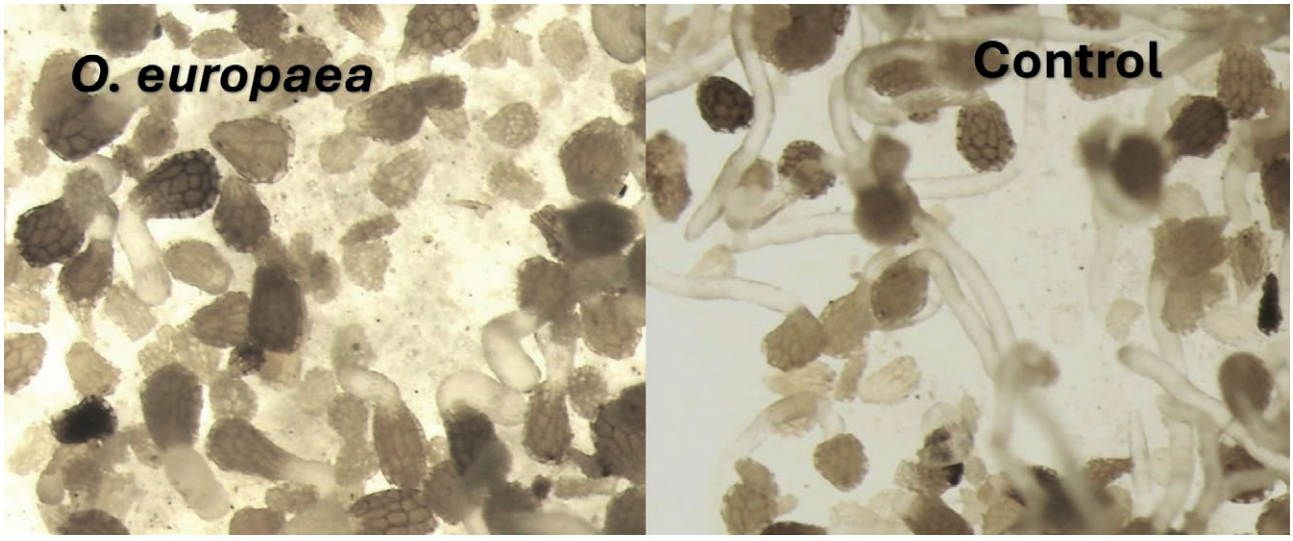

Figure 2. Binocular microscope images showing deformed germ tubes of *Phelipanche ramosa* seeds after treatment with organic extract from *Olea europaea* (left image), compared to the control treatment (right image).

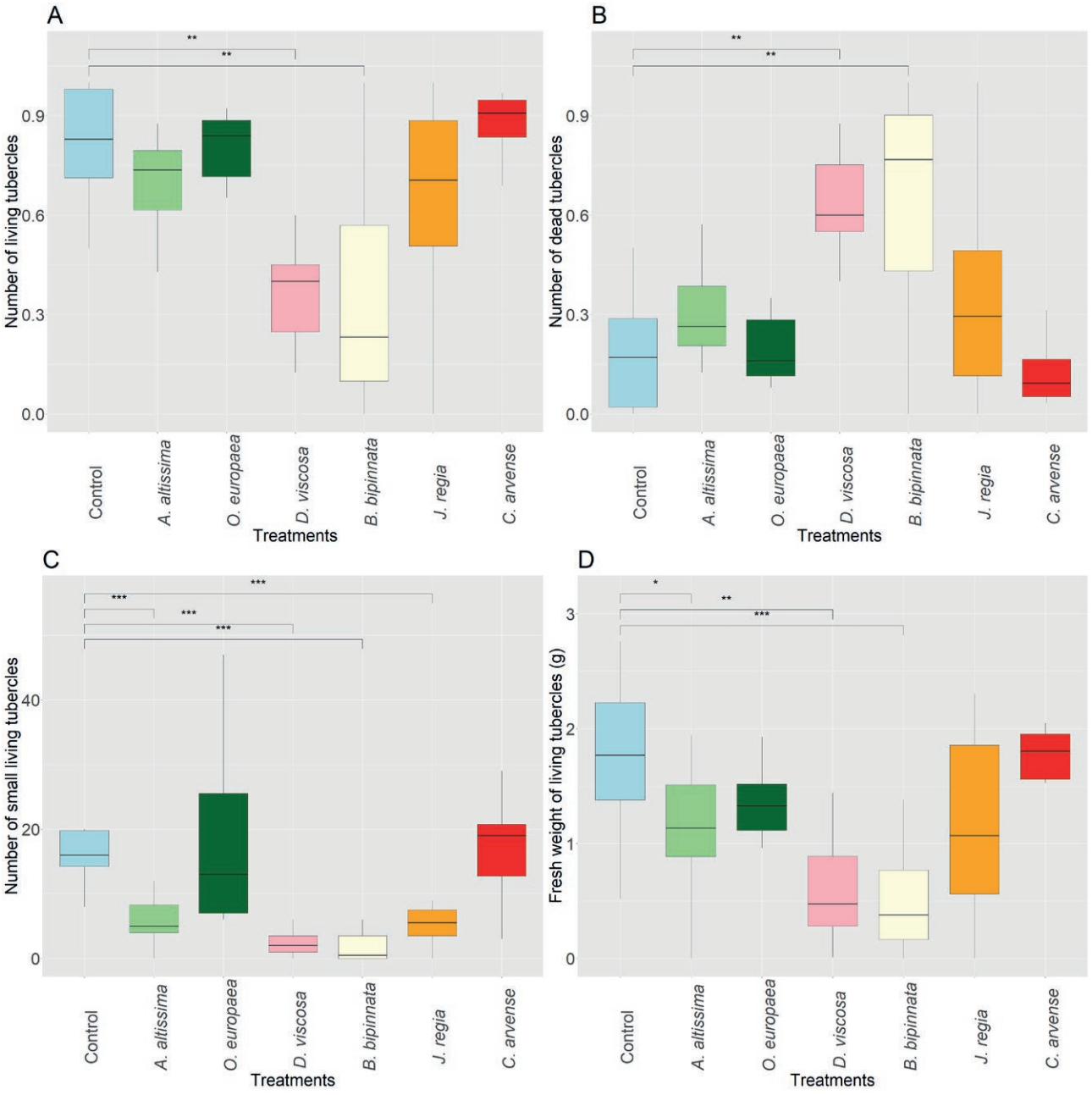

Figure 3. Boxplots of parameters for *Phelipanche ramosa* tubercles after exposure to organic extracts from different plants ("Treatments") in the tomato infectivity test. A, numbers of living *P. ramosa* tubercles; B, numbers of dead tubercles; C, numbers of small living tubercles; D, fresh weights of living tubercles.

After 30 d from the treatment application, the numbers of live *P. ramosa* tubercles was reduced by organic extracts from *B. bipinnata*, *D. viscosa*, *J. regia* and *A. altissima* to be 0.2, 0.4, 0.7, and 0.75, respectively, compared to the control (0.85) (Figure 3 A).

The numbers of dead *P. ramosa* tubercles treated with organic extracts of *B. bipinnata* was the greatest (mean = 0.8), followed by *D. viscosa* (0.6), both of which were greater ($P < 0.05$) than the control (mean = 0.17) (Figure 3 B).

The numbers of small (<0.3 cm^2) *P. ramosa* tubercles were reduced from the treatments of organic extracts from *B. bipinnata* (mean = 2.2), *D. viscosa* (2.7), *J. regia* (5.1), and *A. altissima* (mean =

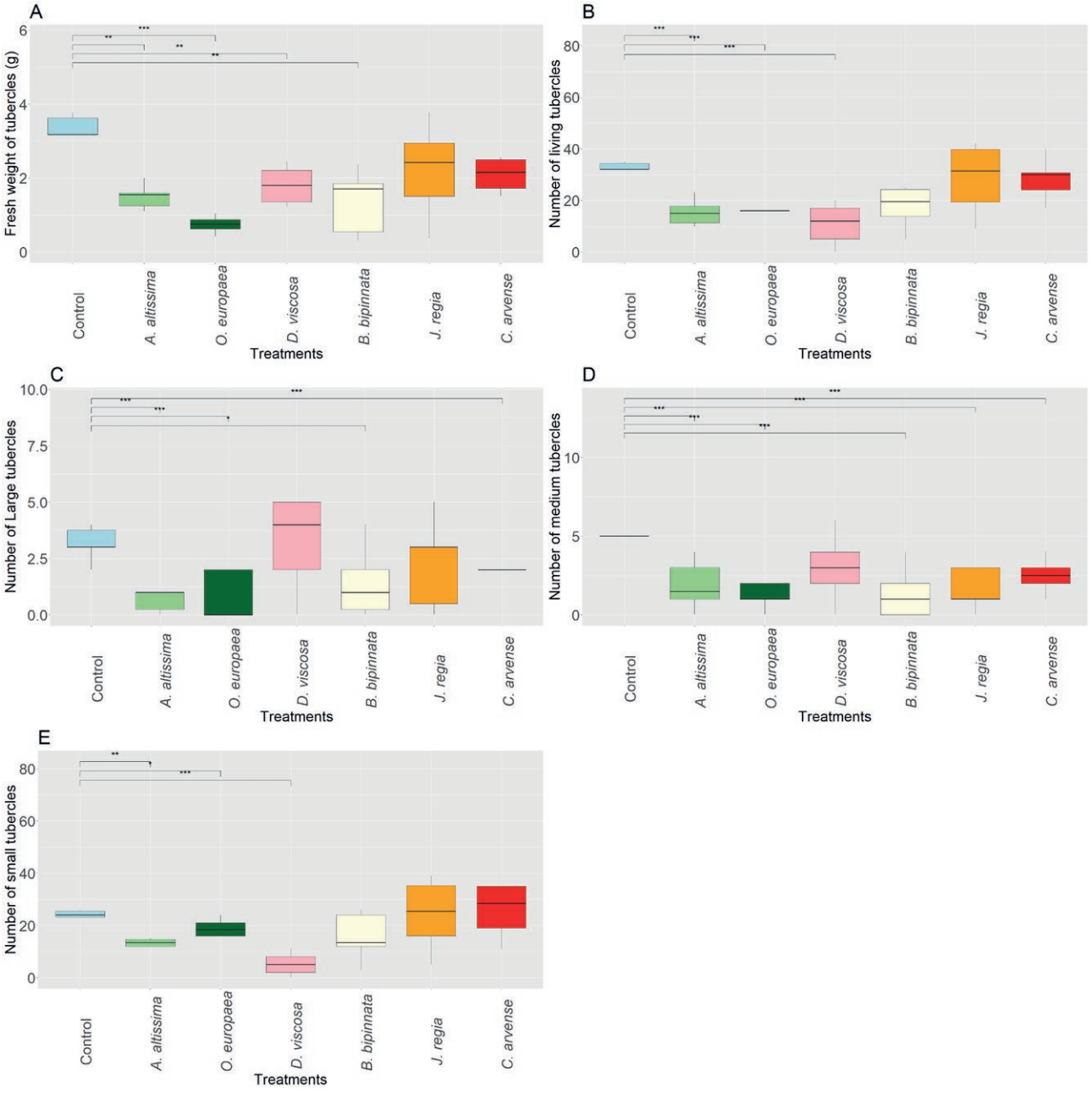

Figure 4. Boxplots regarding the pot experiment carried out by using the organic extracts of the plants. A: fresh weight of living *P. ramosa* tubercles; B: number of living tubercles; C: number of large-size tubercles; D: number of medium-size tubercles; E: the number of small-size tubercles.

5.7), compared with the control (mean = 17.9) (Figure 3 C).

Application of organic extracts reduced mean fresh weights of *P. ramosa* living tubercles to 0.51 g for *B. bipinnata*, 0.56 g for *D. viscosa*, 1.0 g for *A. altissima*, 1.1 g for *J. regia*, and 1.3 g for *O. europaea*, compared to 1.8 g for the control treatment (Figure 3 D).

Pot experiment

Twenty d after application of treatments, fresh weights of *P. ramosa* living tubercles treated with the organic extracts of *O. europaea* (mean = 0.7 g) were less compared to the controls (3.1 g). All the other treatments with *B. bipinnata* (mean = 1.3 g), *A. altissima*

(1.5 g), *D. viscosa* (1.6 g), *C. arvense* (2.0 g), and *J. regia* (mean = 2.2 g) also reduced tubercule fresh weights (Figure 4 A).

Numbers of living tubercles were reduced by applying organic extracts of *D. viscosa* (mean = 11.2), *A. altissima* (14.2), *O. europaea* (14.4) and *B. bipinnata* (24.6), compared with the control (mean = 31.9) (Figure 4 B).

The numbers of tubercles with sizes >0.6 cm^2 were less from the treatments with organic extracts of *A. altissima* (mean = 0.7), *O. europaea* (0.8), and *B. bipinnata* (mean = 1.5), compared to the control (mean = 3.2; Figure 4 C).

Numbers of tubercles with medium size (0.3-0.6 cm^2) were less from all the treatments of organic extracts of *O. europaea* (mean = 1.2), *B. bipinnata* (1.3), *J. regia* (2.6), *A. altissima* (1.8), *C. arvense* (2.4), and *D. viscosa* (mean = 3.1), compared to the control (mean = 5.4; Figure 4 D).

The numbers of small (<0.3 cm^2) *P. ramosa* tubercles were less from all of the treatments with organic extracts of *D. viscosa* (mean = 5.2), *A. altissima* (11.6), and *O. europaea* (mean = 16.3), compared with the control (mean = 23.1; Figure 4 E).

DISCUSSION

This study included bioassays to assess the effectiveness of plant organic extracts for inhibiting seed germination and tubercle development of *P. ramosa*. In the initial germination bioassay, three extracts, those from *A. altissima*, *B. bipinnata*, and *C. arvense*, completely inhibited seed germination at the assessed concentrations. Two other extracts, from *D. viscosa* and *J. regia*, had comparatively lower activity. In the tomato infectivity assessments, *B. bipinnata* and *A. altissima* were consistently toxic to *P. ramosa*, but *C. arvense* had reduced effectiveness compared to the initial seed germination bioassay. This variation indicates a need to explore potential factors contributing to the observed assay differences. In contrast, the *D. viscosa* extract gave stronger inhibition of *P. ramosa* in the tomato infectivity bioassay than in the germination assay.

In the assessment of *O. europaea* and *D. viscosa* extracts in the tomato infectivity assay, *O. europaea* gave less inhibition but longer phytotoxicity, while *D. viscosa* had stronger toxicity but for shorter periods. This possibly explains why the numbers of medium-sized living tubercles were greater from *O. europaea* than *D. viscosa* treatments. The most effective organic extracts against *P. ramosa* in tomato infectivity assay were from *B. bipinnata* and *D. viscosa*, with both extracts giving the least numbers of living tubercles, lowest fresh weights of living tubercles, and greatest numbers of dead tubercles.

In the pot experiment, most of the extracts behaved differently compared to the tomato infectivity assay. This could have been due to the different substrates used in the two assays. In soil, there can be interactions with microorganisms and soil physical and chemical complexes that lead to the decomposition and alteration of biochemicals in organic extracts. This could lead to different effects and functions (positive or negative) of the compounds at the whole system level. In the pot experiment, the host plants were in conditions similar to those of normal growth, and the plants would behave as in field conditions. The extract that showed greatest contrast in effects from the tomato infectivity assay to the pot bioassay was *O. europaea*, having greater activity the pot bioassay than the tomato infectivity assay.

The most effective treatments for reducing numbers of living *P. ramosa* tubercles were *D. viscosa*, *O. europaea*, *A. altissima*, and *B. bipinnata*, although *D. viscosa* and *A. altissima* were less effective in case of the living tubercule fresh weight. The overall performance of these extracts ranged from very inhibitory to mildly inhibitory compared to the experimental control.

Treatment with *D. viscosa* resulted in more large tubercles and fewer small tubercules than from the other treatments. This treatment was probably more effective on small than on large tubercles, and possibly because the active compound was unstable and rapidly degraded in the soil, it could not affect growth of, already well-developed, tubercles that are more dependent on the host plant, and more resistant to the treatment in the surrounding environment. Number of large tubercles also influenced fresh weights of the living tubercles. For example, *O. europaea* and *B. bipinnata* gave lower fresh weights of tubercles than *D. viscosa* and *D. viscosa* gave greater numbers of large tubercles than *O. europaea* and *B. bipinnata*. Extracts from *O. europaea* and *B. bipinnata* gave greater numbers of small tubercles than that from *D. viscosa*.

Activity of extracts from *B. bipinnata* have not been previously investigated for effects species of broomrapes, and allelopathic effects of *B. bipinnata* have been rarely noted. The plant genus *Bidens* is well known for its strong allelopathic activity, which probably arises from a variety of secondary metabolites, including phenolics, saponins, flavonoids, glycosides of flavones, polyacetylenes, terpenes, chalcone glucosides, phenylpropanoid glucosides, and terpenoids (Brandão *et al.*, 1997). The invasive relative from the same genus, *B. pilosa* has been widely studied, and produces many secondary metabo-

lites and allelochemicals active against other plant species. Khanh *et al.* (2009) reported that application of *B. pilosa* biomass suppressed weeds, but this has not been assessed against *P. ramosa*.

Organic extracts of *D. viscosa* were assessed for effects on germination of *P. ramosa* seeds by Serino *et al.* (2021), and our findings agree to the results found by them regarding the germination bioassay of *P. ramosa*, despite that they used biodegradable polymer to preserve release of the extract over long periods to improve the efficacy of the extract. Furthermore, many Other studies have reported inhibitory effects of organic extracts from *D. viscosa* on *P. ramosa* seed germination and tubercle development (Moeini *et al*., 2019; Boari *et al*., 2021). Laboratory investigation showed that *D. viscosa* produced several secondary metabolites, including sesquiterpenoids, flavonoids, and caffeic acids, although in varying concentrations across different plant parts (Grauso *et al*., 2020).

Olea europaea was studied against *P. ramosa* in tomato crops by Qasem (2020), who reported that olive mill wastewater and olive cakes reduced dry weight of *P. ramosa*, with wastewater being most active. Those results indicate possible presence of secondary metabolites able to reduce *P. ramosa* seed germination. However, those results from wastewater and olive cake by-products from olive fruits, while the present study assessed organic extracts from the leaves. Olive fruits and leaves have different secondary metabolite profiles, so the present study results provide expanded knowledge on *O. europaea* phytotoxicity. Research by Di Donna *et al.* (2010) and Kabbash *et al.* (2023) analyzed metabolites produced in *O. europaea* leaves using high performance liquid chromatography and electrospray ionization tandem mass spectrometry. This showed the presence of twelve phenolic compounds in the leaves.

The present study results for *A. altissima* agree with those of Scavo and Mauromicale (2020), who reported that extract from this plant can completely inhibit germination of *P. ramosa*. Strong allelopathic activity lead those authors to extract canthin-6-one (CO) alkaloids and ailanthone which have strong allelopathic activity (Cho *et al*., 2018).

In plastic bags, extracts from *A. altissima*, *C. arvense*, and *J. regia* did not show strong activity as in Petri dish tests. This could be due to the instability of the phytotoxic compounds active against *P. ramosa*. The active compound must be present in appropriate concentrations when *P. ramosa* seeds (already conditioned) are germinating to be effective. If the time of application is too early, the compounds could degrade without affecting *P. ramosa* seed germination. The active compounds must be present during germination, but not after attachment, since the tubercles become more isolated from the environment and are completely dependent on the host plants (Vurro *et al*., 2019).

In conclusion, this study has provided valuable insights into the potential of organic extracts from several spontaneous plants for control of *P. ramosa*. Organic extracts of *B. bipinnata* and *D. viscosa* should be further investigated because of their demonstrated inhibitory effects on *P. ramosa* seed germination and tubercule development. The organic extract of *O. europaea* also reduced *P. ramosa* tubercule fresh weights. Different extraction procedures, without solvents, could be assessed in future experiments, which could be utilized for chemical-free extraction. It would be also advisable to identify the chemical compounds active in suppressing P. ramosa seed germination and tubercle development to better understand the mechanism of action to improve the effectiveness of *P. ramosa* control. Effective plant species should be also tested in field conditions, where they could be directly cultivated and incorporated into soil prior to tomato cultivation. Appropriate application times should be investigated so that the decomposition of these plant materials would timely release the secondary bioactive metabolites into the soil and provides effective broomrape control. This would be an innovative and sustainable approach and could provide intensive solution for combating parasitic weeds.

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AUTHOR CONTRIBUTIONS

Conceptualization, MV, GJC and AB; methodology, MV, AB and GJC; validation, MV, GJC and AB; formal analysis, EE; investigation, EE; resources, GJC, MV and AB; data curation, EE and GJC; writing of original draft, EE; writing after review and editing, MV and GJC; visualization, EE; supervision, GJC, MV and AB; project administration, GJC.

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