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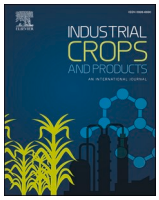

Investigating the phytotoxic potential of *Carlina acaulis* essential oil against the weed *Bidens pilosa* through a physiological and metabolomic approach

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ABSTRACT

Essential oils (EOs) are widely studied as possible candidates for new eco-friendly herbicides for weed management due to their phytotoxicity. In this study we tested the phytotoxic potential of the EO obtained from the roots of *Carlina acaulis* L. (Apiaceae) against the weed *Bidens pilosa* L. This EO, containing 98% of the polyacetylene carlina oxide, showed strong phytotoxic effects on the plant metabolism, such as leaf necrosis, reduction of relative water content and total leaf area, and an increase in the dry weight/fresh weight ratio, suggesting a water status alteration. The EO also damaged the photosynthetic machinery, as evidenced by the significant reduction of the effective quantum yield of photosystem II (Φ_{II}) and the maximum quantum yield of photosystem II (F_v/F_m). In addition, the non-photochemical quenching (Φ_{NPQ}) significantly increased after spraying with *C. acaulis* EO. Damage to photosystem II was further demonstrated through the reduction of manganese and calcium concentrations, possibly due to an alteration in the correct functionality of the Mn_4Ca cluster of the PSII. Metabolomics analysis revealed an accumulation of branched-chain amino acids, such as isoleucine and valine, which is commonly related to osmotic alterations under drought stress situations and a general reduction in sugar content (fructose, glucose, mannose, among others), suggesting reduction of the photosynthetic efficiency too. Overall, these findings suggest *C. acaulis* EO as a promising natural product with phytotoxic potential against weeds that deserves further investigation.

1. Introduction

Plants actively and passively release several specialised metabolites with protective functions into the environment to ensure the necessary nourishment and take advantage of other competing species (Hierro and Callaway, 2021). These compounds spread into the soil, being able to inhibit or limit other species' germination and growth capacities (Xiao et al., 2020). In other words, plants can prevent the growth of potential competitors and gain an advantage in the surrounding environment by producing specialised metabolites harmful to other species. The molecules endowed with these properties are known as allelochemicals and can act by altering seed germination or damaging the interfering weed seedlings' establishment (Hierro and Callaway, 2021). Given their ecological role, these compounds could be considered promising molecules to be employed for the production of botanical herbicides or to be used as a backbone for developing new classes of natural-like herbicides for weed management (Araniti et al., 2015; Berestetskiy, 2023).

In the last decades, weeds have developed two main resistance mechanisms to herbicides: target-site resistance, involving gene mutations that affect herbicide protein targets, and non-target-site resistance, which involves complex interactions of genes from large gene families responsible for reduced herbicide absorption, translocation, degradation, and sequestration. These resistance mechanisms can combine at the individual level, demonstrating weeds' remarkable adaptability to selection pressures (Gaines et al., 2020).

Few new chemical classes of herbicides and molecules with new

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molecular targets remain unchallenged by resistance (Qu et al., 2021). The European Union has implemented restrictions on synthetic substances commonly used in pesticides, resulting in a 60% reduction in available substances for European farmers since 1991 (Directive 91/414/EEC), without adequate replacements. The European Conservation Agriculture Federation promotes the adoption of conservation agriculture, which aims to preserve and optimize natural resources through integrated management of soil, water, biological agents, and external inputs. While herbicides are not entirely prohibited, their rational use and the incorporation of environmentally friendly products such as natural herbicides based on specialized metabolites, are encouraged (Directive 91/414/EEC).

Among different classes of specialized metabolites, polyacetylenes are allelopathic compounds with ecological importance. They are formed in the cytoplasm of plant cells by the polymerisation of malonyl units, leading to an acyl chain that acts as building block of these compounds (Minto and Blacklock, 2008; Negri, 2015). They are characterised by two or more triple bonds in their skeleton and are divided into four main classes according to the classification of Christensen and Brandt (2006). Polyacetylenes are mostly produced in Asteraceae's root system as defensive compounds protecting against microorganisms and parasites or deterring herbivores from feeding (Bohlman et al., 1973; Khanh et al., 2009; Konovalov, 2015). Several studies evidenced their efficacy in inhibiting the germination and growth of seedlings of different competing species (Nishidono and Tanaka, 2022; Stevens, 1986).

Many polyacetylenes are volatile compounds, that can be found as constituents of plant essential oils (EOs), playing several ecological roles (Kobayashi et al., 1980; Quintana et al., 2008). The roots of *Carlina acaulis* L. (Asteraceae) contain around 1% of EO, which is almost made up of the aromatic polyacetylene carlina oxide, reaching 90% or more of the total EO composition (Benelli et al., 2019; Pavela et al., 2021). This molecule is formed by one benzyl and one furan moieties linked to each other by a triple bond (Fig. 1).

This EO was recently reported to be one of the most active EOs ever tested against arthropod pests and vectors (Pavela et al., 2020; Benelli et al., 2021, 2022; Rizzo et al., 2021; Kavallieratos et al., 2022a; Spinozzi et al., 2023a) and phytophagous nematodes (Ntalli et al., 2023) of economic importance, being a promising candidate to be used in insecticidal and acaricidal formulations (Benelli et al., 2020; Pavela et al., 2021; Kavallieratos et al., 2022b).

Plant EOs are also known to be promising bio-herbicides due to their ability to inhibit seed germination and seedlings growth, causing oxidative stress and disrupting the photosynthetic machinery (Synowiec et al., 2019; Pouresmaeil et al., 2020, 2022; Verdeguer Sancho et al., 2020; Werrie et al., 2020). Indeed, many EOs and extracts belonging to different plant species have already been assayed against troublesome worldwide weeds such as *Bidens pilosa* L. with promising results regarding the possible development of new natural herbicides (Pergo et al., 2008; Abd El-Gawad, 2016). For example, extracts of *Bacharis* spp. have shown strong phytotoxic effect against *B. pilosa* diaspores, being suggested as candidates for the development of new herbicides against *B. pilosa* invasion in crop fields (Dias et al., 2017). *Bidens pilosa* (Asteraceae) is an annual dicot herbaceous species native to tropical America and is considered a widely problematic weed in many ecosystems due to its resistance to conventional herbicides, strong adaptability and high invasiveness (Takao et al., 2011; Chauhan et al., 2019). Since the lack of knowledge on the phytotoxicity of this natural product, in this work we have tested for the first time the phytotoxicity of *C. acaulis* EO on *B. pilosa* adult plants. Furthermore, the EO effects on the photosynthetic machinery and metabolomic profile were evaluated as well.

2. Materials and methods

2.1. Carlina acaulis essential oil hydrodistillation

The dry roots of wild *C. acaulis* (batch no C-130320290420, produced in 2019) were derived from an Albanian accession and acquired from Minardi & Figli S.r.l. (Bagnacavallo, Ravenna, Italy; https://www.minardierbe.it). One kg of plant material was soaked overnight in 7 L of distilled water in a glass flask of 10 L of volume inserted into a mantle system (Falc Instruments, Treviglio, Italy). The day after it was subjected to hydrodistillation for 6 h and the EO was recovered through a Clevenger-type glass apparatus (Benelli et al., 2021; Rosato et al., 2021). The EO yield, in %, was estimated on a dry weight basis.

2.2. Chemical characterisation of Carlina acaulis essential oil

The EO was diluted 1:100 in *n*-hexane and then analysed with an Agilent 8890 gas chromatograph (GC) furnished with a single quadrupole 5977B mass spectrometer (Santa Clara, California, USA). The system had an autosampler PAL RTC120 (CTC Analytics AG, Zwingen,

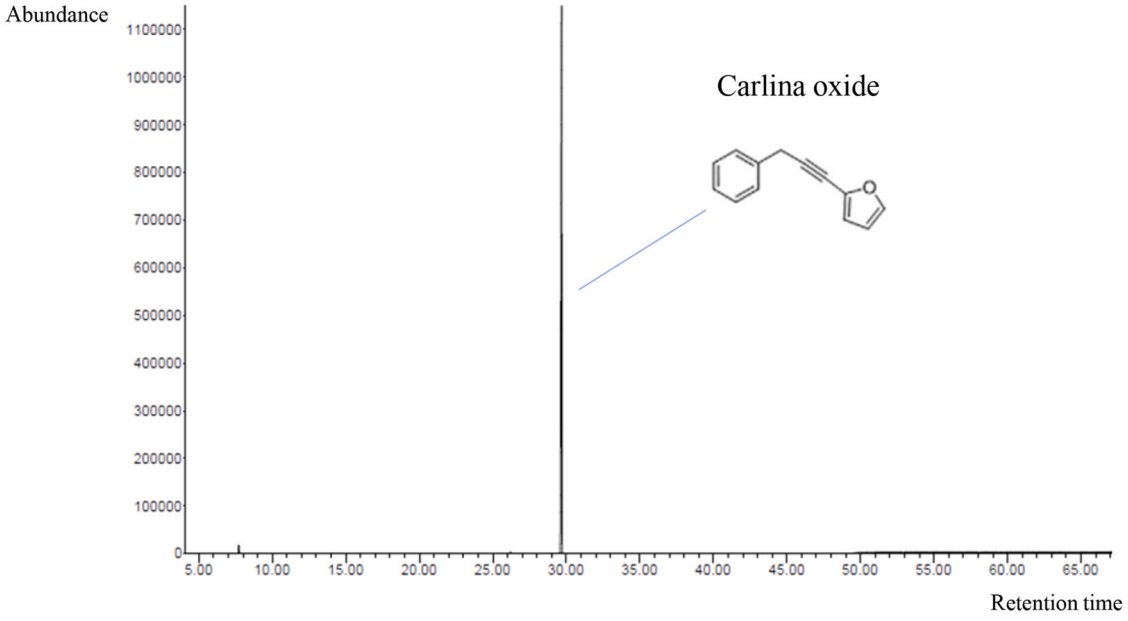


Fig. 1. Chromatogram obtained from the *Carlina acaulis* essential oil GC-MS analysis.

Switzerland). For the separation, an HP-5 MS capillary column (30 m, 0.25 mm i.d., 0.1 μm film thickness; 5% phenylmethylpolysiloxane, Agilent) was used and programmed as follows: 60 °C for 5 min, then raised up to 220 °C at 4 °C/min, finally up to 280 °C at 11 °C/min and held for 15 min. The temperature of injector and detector was 280 °C. The mobile phase was He (99.9%) with a flow of 1 mL/min. After dilution in *n*-hexane, the EO sample was injected (2 μL) in split mode (1:50). The scan range for acquisition of peaks was 29–400 *m/z* using the electron impact (EI, 70 eV) mode. The MSD ChemStation software (Agilent, Version G1701DA D.01.00) and the NIST Mass Spectral Search Program for the NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library v. 2.3 were used to analyze data. Carlina oxide was identified using the standard previously isolated in our laboratory (Benelli et al., 2021), whereas the other compounds by the interactive combination of the temperature-programmed retention indices (RIs) and mass spectra (MS) with respect to those of ADAMS, NIST 17, and FFNSC3 libraries. The RI was calculated using a mix of *n*-alkanes (C_8-C_{30}, Supelco, Bellefonte, CA, USA) according to the Van den Dool and Kratz (1963) formula. Relative area percentages were obtained by peak area normalization without using correction factors.

2.3. Phytotoxic experiments on Bidens pilosa

Bidens pilosa seeds were sown in pots containing professional commercial soil (orticole alveolo TecnoGrow, Tercomposti, Italy, Calvisano, BS) and grown in greenhouse conditions (25 °C ± 5, 12/12 h) until they reached the phenological stage of 4 true leaves. Four plants per pot were grown, and five pots per treatment were used for the experiment. The EO emulsions were prepared with ethanol in the following concentrations: 4, 8, 12, and 20 μL/mL, adding the natural based adjuvant Osmosis® (Delbon, France) at commercial concentrations. The same amount of Osmosis® and ethanol concentrations were used for preparing the control concentration (0 μL/mL). These concentrations were estimated based on previously published papers about EO phytotoxicity on weeds (Verdeguer Sancho et al., 2020; Bellache et al., 2022). Plants were sprayed only once with 10 mL of the EO emulsion until the leaves were covered with tiny droplets before the point of runoff, and then different measurements were done at 0, 6, 24, and 48 h.

2.4. Chlorophyll a fluorescence measurements

The chlorophyll *a* fluorescence measurements were carried out before the EO spraying and after 6, 24, and 48 h of EO treatment to evaluate its early effects on *B. pilosa* plants. The measures were carried out in situ using the MultispeQ fluorimeter v2.0 (PhotosynQ, East Lansing, MI, USA). The rapid information dense experimental sequence (RIDES) protocol ("Photosynthesis RIDES 2.0"), available on the PhotosynQ platform [https://photosynq.org/, (Kuhlgert et al., 2016)] was run to measure the effective quantum yield of the photosystem II photochemical reactions (Φ_{II}), the regulated energy dissipation in the form of heat (Φ_{NPQ}), and the non-regulated energy dissipation (Φ_{NO}, fluorescence emitted). The maximum quantum efficiency of photosystem II (F_v/F_m) was also estimated after leaving the plants in darkness for 20 min to have all the reaction centres opened. For this measure, we used the MultispeQ v2.0 to run the "F_v/F_m in the dark" protocol, also linked to the PhotosynQ platform [https://photosynq.org/]. We randomly selected five plants per treatment for all the chlorophyll *a* fluorescence measurements.

2.5. Post-harvest measurements: leaf area, plant biomass, relative water content (RWC)

After 48 h of EO treatment, the plant material was collected and prepared to take the following measures:

- Total leaf area was calculated with ImageJ, by selecting three plants per treatment and photographing every leaf. All data were expressed as a percentage of the control.
- Dry (DW) and fresh weights (FW) were estimated on the aerial parts of three plants, which were weighed immediately after collection to obtain the FW and then oven-dried at 60 ºC for 72 h to obtain the DW.
- The relative water content (RWC) was evaluated on three plants per treatment, which were weighed and immersed in ultrapure water for 24 h under light. Then, turgid weight (TW) and DW were obtained by weighing plants again and over-drying them at 70 ºC for 72 h. The following equation was used to calculate the RWC value:

$$RWC = [(FW - DW) / (TW - DW)] \times 100$$ (Pieczynski et al., 2013).

2.6. ICP-MS ionomic analysis

For ionomic analysis, a pool of leave material was prepared for each EO concentration tested after 48 h of EO exposition and then dried at 70 ºC for 72 h. One hundred mg per treatment in three replications were selected and transferred to Teflon tubes filled with 10 mL of 65% (v/v) HNO_3 to be digested by a microwave digester system (MULTIWAVE-ECO, Anton Paar Italia Sfl., Rivoli, Italy). Then, two steps of power ramp were applied (Step 1: at 500 W in 10 min maintained for 5 min; Step 2: at 1200 W in 10 min maintained for 15 min). Mineralised samples were transferred into polypropylene test tubes and diluted 1:20 in Milli-Q water after 20 min of cooling. The concentration of the considered elements (Na, Mg, Al, K, Ca, Cr, Mn, Fe, Zn, Se, Cd, and P) were measured by inductively couple plasma-mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS, Bruker Daltonik GmbH, Leipzig, Germany). An aliquot of a 2 mg/L internal standard solution (^{72}Ge, ^{89}Y, and ^{159}Tb) was added to both samples and multi- and single- (for As and P) element calibration standards to obtain a final concentration of 20 μg/L. A collision-reaction interface (CRI) with an H_2 flow of 70 mL/min was used to remove possible polyatomic interferences (Orasen et al., 2019). We compared the mineral contents between untreated and treated plants with 4, 8, 12, and 20 μL/mL of *C. acaulis* EO emulsion for 48 h.

2.7. GC-MS-driven untargeted metabolomics analysis

The untargeted metabolomics analysis was carried out on *B. pilosa* aerial parts using the protocol proposed by Lisec et al. (2006) and modified by Misra et al. (2020). After extraction and derivatisation, the samples spiked with ribitol as internal standard (0.2 mg/mL) were injected into an Agilent gas chromatograph apparatus (GC 7890 A) coupled to a single quadrupole mass spectrometer (MS 5975 C INERT XL MSD) and a CTC ANALYTICS PAL autosampler. A MEGA-5MS (30 m × 0.25 mm × 0.25 μm + 10 m of pre-column) column (MEGA s. r.l., Milan, Italy) was used for sample chromatography, and ultra-pure helium (6.0) with a flow rate of 1 mL/min was used as the gas carrier. The bioinformatics analysis of the chromatograms (sample alignment, deconvolution, peak extraction and annotation) were carried out using the open-source software MS–DIAL ver. 4.49. Peak intensities were extracted, and the metabolites were putatively annotated using an in-house library built using open-source, and publically available EI spectral libraries as previously described by Misra et al. (2012). Metabolites annotation was carried out at level 2 (identification based on the spectral database) and level 3 (only compound groups were known, e.g. specific ions and RT regions of metabolites) following the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sansone et al., 2007).

2.8. Statistical Analysis

All experiments were carried out with a completely randomised design using different replications depending on the experiment. Data

were tested for normality and homogeneity or heterogeneity and then analysed through one-way ANOVA using the Tukey's test or Tamhane's T2 test as post-hoc, respectively ($p \leq 0.05$). Those experiments with three replications were analysed through Kruskal-Wallis' test ($p \leq 0.05$). Metabolomic data were analysed using the software Metaboanalyst 5.0. as reported by Wagner et al. (2022). Lowess normalisation was applied on MS-DIAL software using the internal standard Ribitol. Then, dataset was charged on Metaboanalyst 5.0 and 'Log$_{10}$ transformation' and 'Pareto-scale' were applied. Data were classified and discriminated through the Principal Component Analysis (PCA) and the Orthogonal Partial Least-Discriminant Analysis (OPLS-DA). The univariate *t*-test ($p \leq 0.05$) was used to analyse statistical differences among metabolites from control and EO-treated plants. The False Discovery Rate (FDR) was applied to the nominal *p*–values. Finally, an enrichment and pathway analysis was carried out using the Metaboanalyst 5.0 tools to identify the main altered pathways. All raw data are reported in Supplementary Material, Table S1.

3. Results

3.1. *Carlina acaulis* essential oil content and composition

The hydrodistillation of the *C. acaulis* roots produced a yellow EO with a yield of 0.73% (density of 1.063 g/mL). The GC-MS analysis was performed for the characterisation of the EO and the determination of its chemical composition. The chromatogram obtained from the analysis (Fig. 1) showed the presence of one major constituent accounting for 98.8% of the total composition, corresponding to the polyacetylene carlina oxide. This content is in accordance with data obtained in previous studies (Benelli et al., 2022; Kavallieratos et al., 2022a, 2022b; Spinozzi et al., 2023b). Moreover, benzaldehyde and *ar*-curcumene were minor components, accounting for 0.99 and 0.09% of the EO, respectively.

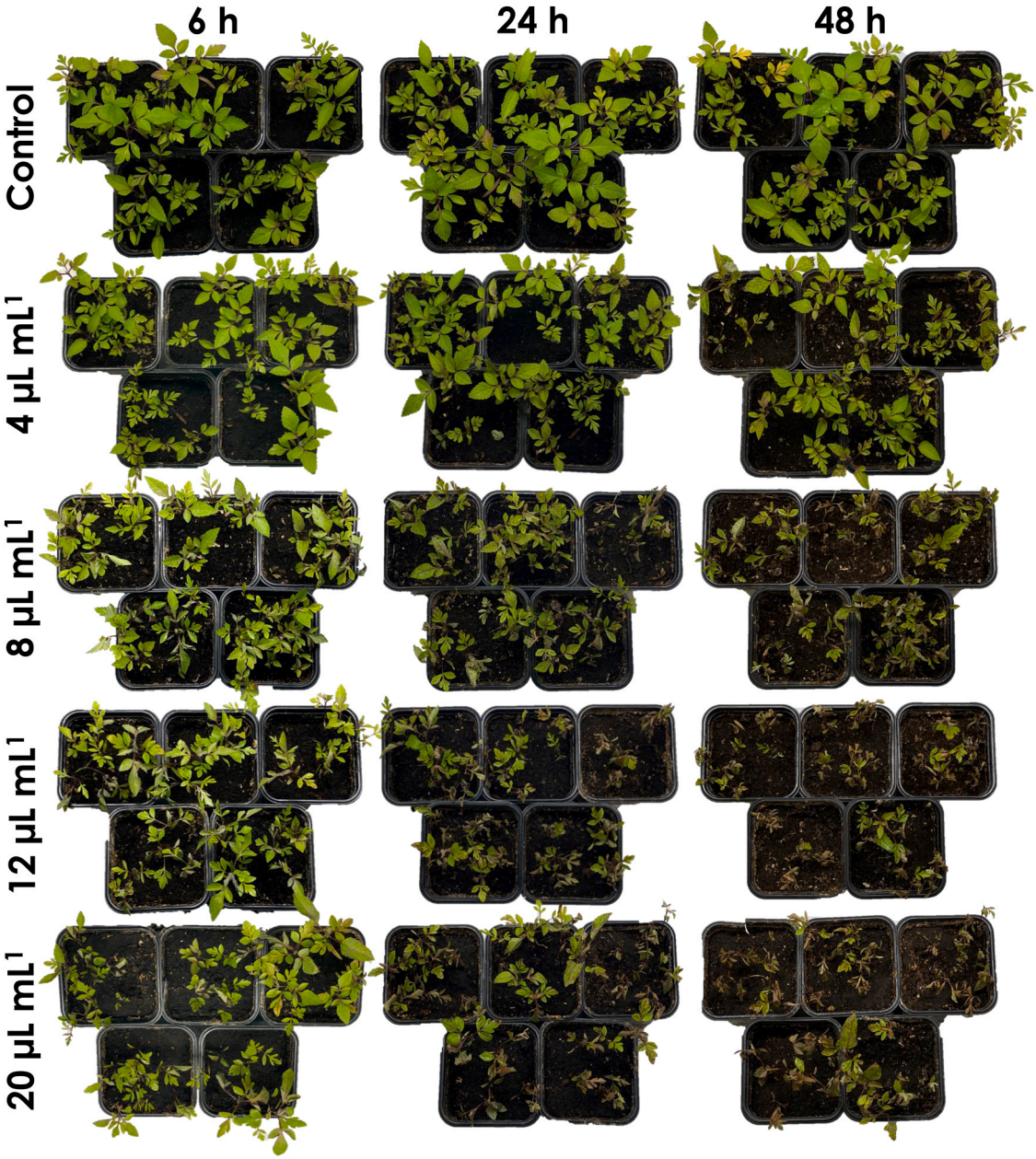


Fig. 2. Phenotypical effects induced on *Bidens pilosa* adult plants after 6 h, 24 h, and 48 h of *Carlina acaulis* essential oil exposition. The following concentrations were used: 4 µL/mL, 8 µL/mL, 12, µL/mL, and 20 µL/mL.

3.2. Phytotoxic potential of Carlina acaulis essential oil

Bidens pilosa adult plants were sprayed once with EO emulsion to test its phytotoxic potential. Visual and internal early effects caused by the EO were evaluated after 6, 24, and 48 h exposition.

Leaf damages were already observable 6 h after spraying (Fig. 2) and became more severe over time. Treated leaves experienced signs of dryness or were completely dried out and died at the highest concentrations tested (12 and 20 μL/mL) after 24 h. Similar effects were also observed in plants treated with 8 μL/mL concentrations after 48 h of treatment (Fig. 2).

The *C. acaulis* EO damaged the leaves and reduced the total leaf area after 48 h. Plants showed a significant downward trend for all concentrations tested (Fig. 3A). The lowest EO concentration assayed (4 μL/mL) caused a 40% reduction compared with control plants, and this difference increased as EO concentration increased, reaching around 70% reduction for the highest concentrations tested (12 and 20 μL/mL).

RWC significantly decreased for those *B. pilosa* plants sprayed with 8, 12, and 20 μL/mL of *C. acaulis* EO, reaching reductions of 21%, 25%, and 20%, respectively, when compared with untreated plants (Fig. 3B).

The dry weight/fresh weight ratio (DW/FW) demonstrated that *B. pilosa* plants were dried out by *C. acaulis* EO because the ratio showed an upward trend compared with control as EO concentration increased. The DW/FW showed a 20, 40, 40, and 80% of increment for 4, 8, 12, and 20 μL/mL, respectively, compared with *B. pilosa* untreated plants (Fig. 3C). Both DW and FW appeared reduced (Fig. 3D and E, respectively), reaching a 60% reduction of DW for 12 μL/mL (Fig. 3D) and a 75% of reduction of FW for 20 μL/mL(Fig. 3E).

After 48 h, plant material was collected from those plants exposed to the lowest EO concentration tested (4 μg/mL) to conduct the ionomics analysis. Results revealed that *C. acaulis* EO significantly reduced Na, Mg, Ca, Al, Cd, and Mn levels. However, K, P, and Se significantly increased their levels compared with control plants (Table 1).

3.3. Effects of Carlina acaulis essential oil on PSII parameters

After monitoring the photosynthetic parameters at 0, 6, 24, and 48 h of treatment, we observed that Φ_{II}, Φ_{NPQ}, Φ_{NO} and F_v/F_m showed similar behaviour for all concentrations tested. The efficiency of PSII (Φ_{II}) was progressively reduced as *C. acaulis* EO concentration increased. Significant differences with control plants were obtained after 6 h of EO exposition for 8, 12, and 20 μL/mL, whereas all concentrations tested showed significantly lower Φ_{II} than control after 48 h (Fig. 4A). Simultaneously, the maximum PSII efficiency in darkness (F_v/F_m) showed a downtrend as time and EO concentration increased during the experiment. The F_v/F_m value was significantly lower for 8, 12, and 20 μL/mL treated plants after 6 h of EO exposition, while all the concentrations showed damage on photosystem II' antenna complex after 24 and 48 h EO exposition (Fig. 4D).

The EO similarly affected the non regulated emission of energy in excess under the form of fluorescence (Φ_{NO}), which significantly decreased compared with control plants. Significant differences were found for 4, 12 and 20 μL/mL after 6 and 24 h, and for 12 and 20 μL/mL after 48 h of *C. acaulis* EO exposition (Fig. 4B).

On the contrary, the non-photochemical quenching (Φ_{NPQ}) was higher than control plants for all the concentrations tested after spraying with *C. acaulis* EO. This upward trend was consistent in time, showing significant differences for 8, 12, and 20 μL/mL after 6 h, and for 4, 12, and 20 μL/mL after 48 h EO exposition (Fig. 4C).

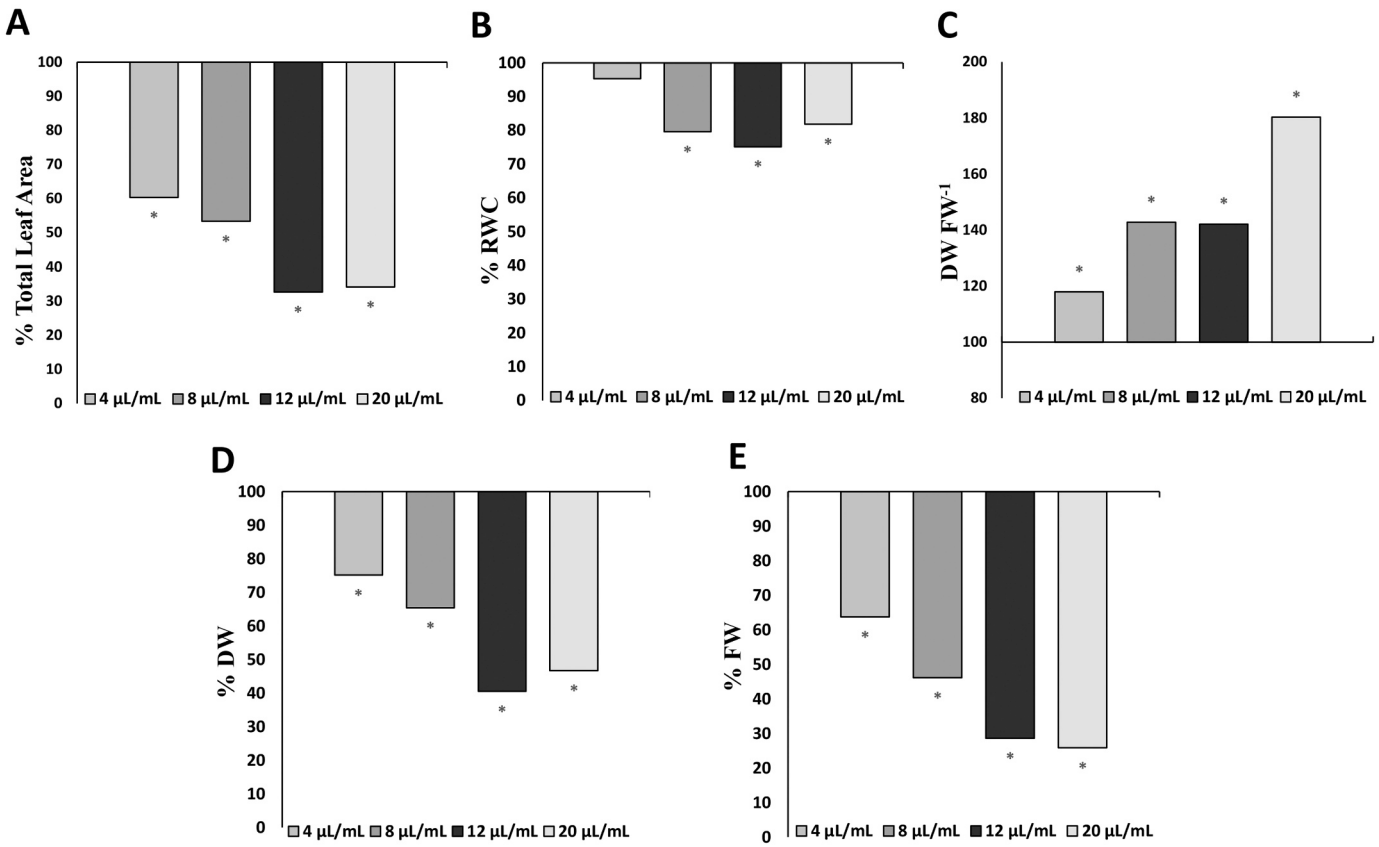


Fig. 3. (A) Total leaf area; (B) Relative water content (RWC); (C) Dry weight / Fresh weight ratio (DW/FW); (D) Dry weight (DW); and (E) Fresh weight (FW) of *Bidens pilosa* plants exposed to different *Carlina acaulis* essential oil concentrations (0, 4, 8, 12, and 20 μL/mL) for 48 h. Data are expressed in percentage of the control. Asterisks indicate statistical differences from respect to the control after Kruskal-Wallis' test; * $p < 0.05$. N = 3.

Table 1
Mineral concentrations of *Bidens pilosa* adult plants after 48 h of treatment with 0, 4, 8, 12, and 20 μg/mL of *Carlina acaulis* essential oil.

Element (μg/μgDW)	Treatment with *C. acaulis* EO (4 μg/mL)				
	0	4	8	12	20
K	28099.33 ± 914.57	31842.43 ± 325.78 *	31532.62 ± 743.03 *	31709.27 ± 437.03 *	30647.31 ± 1004.6 *
P	7419.94 ± 158.28	7881.90 ± 760.18	8551.05 ± 577.72 *	8176.64 ± 627.02	9592.04 ± 423.05 *
Na	104.23 ± 2.89	102.31 ± 2.95	178.83 ± 14.42 *	65.17 ± 0.69 *	70.70 ± 3.58 *
Mg	3649.64 ± 157.73	3624.42 ± 94.06	3794.76 ± 124.76	3500.44 ± 24.80	3351.26 ± 78.93 *
Fe	169.97 ± 13.72	174.51 ± 6.82	197.93 ± 7.91 *	173.27 ± 7.09	159.07 ± 14.60
Ca	20698.96 ± 1270.13	20169 ± 618.64	20029.34 ± 818.87	18908.18 ± 584.33	17098.22 ± 395.33 *
Al	24.68 ± 2.33	8.57 ± 3.06 *	12.27 ± 4.12 *	8.05 ± 2.30 *	9.56 ± 2.68 *
Cr	0.14 ± 0.19	0.53 ± 0.09 *	0.51 ± 0.11 *	0.55 ± 0.28	0.53 ± 0.27
Mn	252.97 ± 15.57	229.74 ± 5.27 *	190.06 ± 14.62 *	198.99 ± 8.61 *	202.96 ± 5.73 *
Se	0.06 ± 0.1	0.47 ± 0.29	0.95 ± 0.16 *	0.65 ± 0.23 *	0.34 ± 0.22
Cd	0.73 ± 0.05	0.59 ± 0.02 *	0.62 ± 0.02 *	0.72 ± 0.01	0.53 ± 0.09 *
Zn	66.47 ± 4.67	75.51 ± 2.99 *	75.62 ± 2.35 *	76.49 ± 2.03 *	59.29 ± 0.5 *

Data are expressed in μg ion / g dry weight of each ion (parts per million). Asterisks indicate significant differences after Kruskal-Wallis' test compared to untreated plants. * $p < 0.05$. N = 3.

3.4. Effects of Carlina acaulis essential oil on plant metabolome

To obtain more insights into the metabolomic changes caused by the *C. acaulis* EO on *B. pilosa* adult plants, a GC/MS-driven untargeted metabolomic analysis was carried out. The analysis compared plants treated with 4 μL/mL of *C. acaulis* EO and untreated plants after 48 h of EO exposition. We have chosen this concentration and time of exposure focusing on the effects of the EO on photosynthetic parameters, particularly F_v/F_m and Φ_{II}.

The analysis identified 444 compounds, of which 122 were putatively annotated, and 322 were considered unknown. However, after discarding false annotated metabolites, a total of 97 metabolites were putatively annotated. Then, the data were normalised, and these annotated metabolites were statistically analysed through Metaboanalyst 5.0.

Data were then analysed through unsupervised (principal component analysis - PCA) and supervised (ortho partial least square discriminant analysis - OPLS-DA) multivariate analysis. In particular, PCA was used for exploratory data analysis and dimensionality reduction, identifying and underlying structure of the data by finding the linear combinations of variables (principal components) that explain the most variation in the data without considering any class of information. On the other hand, OPLS-DA is a supervised method used to classify samples into different groups based on their measured variables.

The Unsupervised Principal Component Analysis (PCA) was performed by comparing untreated and EO-treated plants. The PCA score plot, built on the first (PC1) and second component (PC2), explained a total variance of 83.1% and revealed a clear separation between sample groups (Fig. 5A). PC1 explained 64.2%, whereas PC2 explained 18.9% of the total variance.

Successively, data were analysed through the supervised Orthogonal Partial Least-Discriminant Analysis (OPLS-DA) and a complete separation between groups (control and treatment plants) was achieved, explaining a total variance of 73% (Fig. 5B). The PLS-DA model was characterised by Q^2 and R^2 accuracy were higher than 0.9 and a p value ≤ 0.05 (Table S1).

OPLS-DA derived (VIP) scores (built on the metabolites with a VIP score higher than 1.2) pointed that isohexonic acid, fumaric acid, allose, D-(-)-ribose, quinic acid, shikimic acid, tagatose, D-(+)-mannose, D-(-)-fructose and glucose, among others, were those characterised by the highest VIP scores (Fig. 5C). In particular, isohexonic acid, citric acid, *n*-acetyl-serine, *cis*-aconitic acid, isoleucine, and 2-coumaric acid were significantly accumulated in treated plants. In contrast, all the other metabolites identified by a high VIP score (fumaric acid, allose, ribose, quinic acid, shikimic acid etc.) were significantly reduced (Fig. 5C).

A *t*-test analysis was also performed, revealing 62 significantly altered metabolites among untreated and EO-treated plants (the complete list of these metabolites is available in Table S1). These significantly affected metabolites mainly belong to the class of amino acids (alanine, GABA, isoleucine, and valine, among others), amines (tyramine, ethanolamine, hydroxylamine, and methylethanolamine), sugars (fructose, glucose, mannose, and ribose, among others), sugar acids, amino sugars, sugar alcohols (ribonic acid, acetylmannosamine, hexitol and iditol, among others), organic acids (citric acid, fumaric acid, glutaric acid, and malic acid, among others), etc. (Table 2).

Except for alanine and arginine, the rest of the amino acids increased their content after *C. acaulis* EO treatment. On the contrary, except for xylose and galactonic acid, all sugars, sugar acids, amino sugars and sugar alcohols were inhibited by the *C. acaulis* EO treatment. Regarding organic acids, aconitic acid, citric acid, glutaric acid, and threonic acid were stimulated whereas the rest significantly reduced their concentration compared with untreated plants after *C. acaulis* EO exposition.

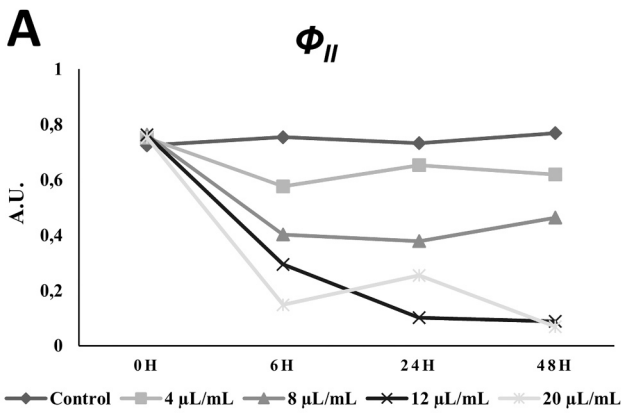
KEGG-based pathway analysis was also carried out using the "MetPa" module of Metaboanalyst to analyse *C. acaulis* EO effects on pathways and networks. The analysis showed that the treatment significantly altered 41 pathways. However, only 16 showed an impact higher than 0.1 (Table 3). Among them, alanine aspartate and glutamate metabolism, glycine serine and threonine metabolism, starch and sucrose metabolism, and TCA cycle were the most impacted by the *C. acaulis* EO treatment, pointing out an impact higher than 0.3 (Table 3).

4. Discussion

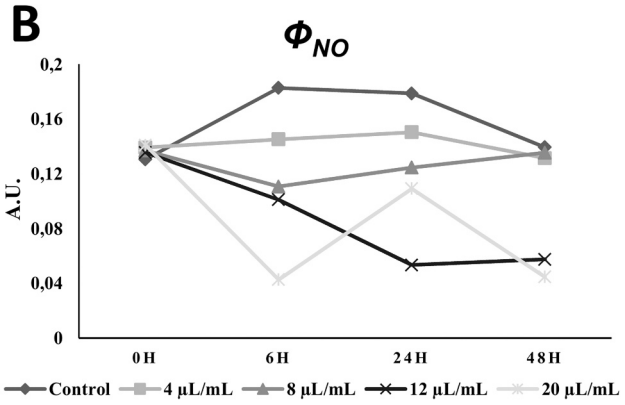
It is well known that the biological activities of *C. acaulis* EO are mainly linked to the presence of carlina oxide as the main constituent (Benelli et al., 2022; Spinozzi et al., 2023a). This compound belongs to the chemical class of polyacetylenes, which have already been reported to be phytotoxic agents. For instance, lachnophylum ester, matricaria ester (Kobayashi et al., 1974), *cis*-dehydromatricariaester (Kawazu et al., 1969), dehydromatricaria lactone (Ichihara et al., 1978), and *α*-terthienyl (Campbell et al., 1982) are just some of the numerous polyacetylenes showing phytotoxic activity (Quintana et al., 2008). Despite the large number of polyacetylenes reported, the chemical moieties needed to display this biological activity are still not understood, as the mechanism of action of the phytotoxicity too (Quintana et al., 2008). Probably, their biological activity could be strongly linked to their sensitivity to sunlight or UV wavelengths (Campbell et al., 1982), but this aspect should be further investigated.

This work tested the effects of *C. acaulis* EO on *B. pilosa* well-established seedlings to evaluate the phytotoxic potential of this EO against weeds. *Carlina acaulis* EO, as also confirmed by the increase in DW/FW, showed strong effects, highlighting its capacity to induce dehydration or cause leaf necrosis. This DW/FW increment was also observed by Araniti et al. (2018) after *Origanum vulgare* L. EO treatment on *Arabidopsis thaliana* (L.) Heynh. seedlings, and by Díaz-Tielas et al. (2014) after *trans*-chalcone treatment, suggesting water loss. Therefore, the significant reduction in RWC after *C. acaulis* EO exposure corroborates that plants could experience an alteration of their water status as a consequence of the treatment.

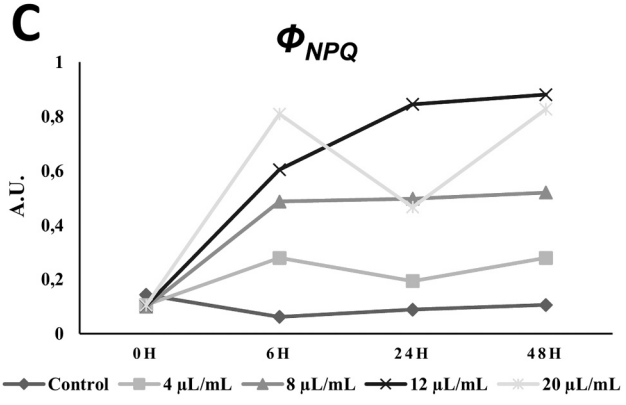
The chlorophyll *a* fluorescence parameters were monitored during EO treatment to check possible alterations in the photosynthetic machinery. After EO exposition, treated plants experienced a significant



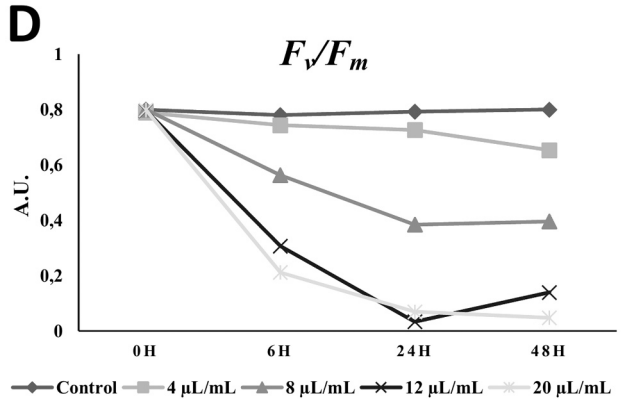
	Φ_{II}			
Hour	4 µL/mL	8 µL/mL	12 µL/mL	20 µL/mL
0				
6		-	-	---
24		---	---	---
48	-	-	-	-



	Φ_{NO}			
Hour	4 µL/mL	8 µL/mL	12 µL/mL	20 µL/mL
0				
6	---			---
24	-		-	-
48			--	---



	Φ_{NPQ}			
Hour	4 µL/mL	8 µL/mL	12 µL/mL	20 µL/mL
0				
6		+	+	+++
24			+++	
48	+		+++	+



	F_v/F_m			
Hour	4 µL/mL	8 µL/mL	12 µL/mL	20 µL/mL
0				
6		-	--	-
24	-	--	-	--
48	--	--	--	-

Fig. 4. Mean values of (A) φ_{II} (effective quantum yield of photosystem II); (B) φ_{NO} (non-regulated energy dissipation; fluorescence emission); (C) φ_{NPQ} (regulated energy dissipation in the form of heat), and (D) F_v/F_m (maximum quantum yield of dark-adapted photosystem II) in *Bidens pilosa* plants exposed to different *Carlina acaulis* essential oil concentrations (0, 4, 8, 12, and 20 µL/Ml) for 0, 6, 24, and 48 h. Tables in the right show the statistical significance differences compared with to untreated plants (+, positive difference; -, negative difference; + or -, $p < 0.05$, ++ or –, $p < 0.01$, +++ or —, $p < 0.001$). Mean values of φ_{II}, φ_{NO} and φ_{NPQ} are expressed in arbitrary units (A.U.). N = 5.

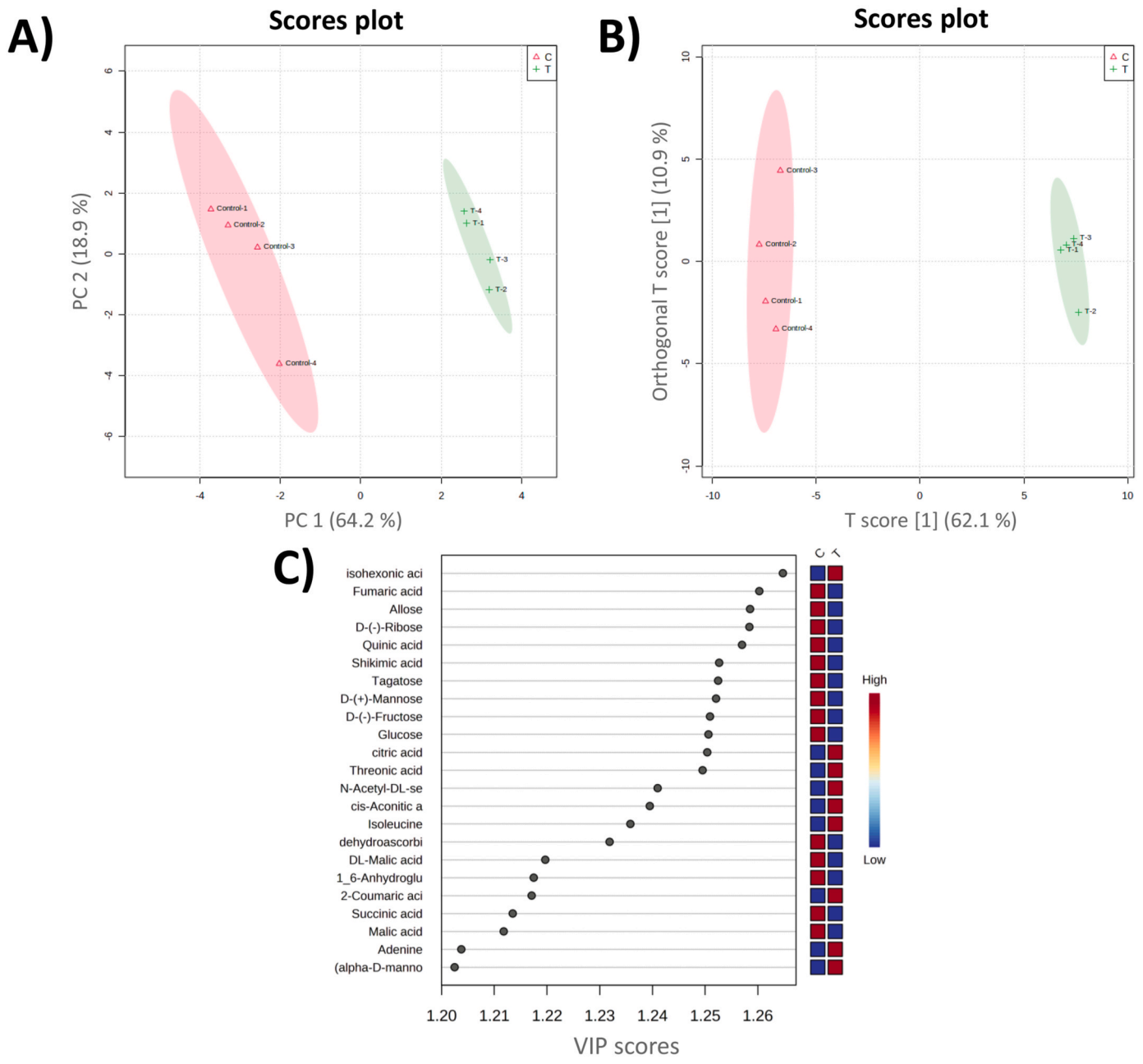


Fig. 5. Graphical representation of (A) Principal component analysis (PCA) and (B) Orthogonal Partial Least-Discriminant Analysis (OPLS-DA) of the metabolomic changes on *Bidens pilosa* after 48 h treatment with 4 μL/mL *Carlina acaulis* essential oil. Separation between groups metabolome is represented through colours. (C) Variable importance of projection (VIP) features for control and treated plants after Ortho-PLS-DA analysis. C (control plants; red colour) and T (EO-treated plants; green colour). N = 4.

reduction of the effective quantum yield of photosystem II (Φ_{II}) and maximum quantum yield of photosystem II (F_v/F_m), suggesting possible physical damage at the PSII' antennae complex and causing a decrease of the photosynthetic efficiency (Sánchez-Moreiras et al., 2020). Indeed, similar results were observed recently by Álvarez-Rodríguez et al. (2023) after harmaline treatment on *A. thaliana* adult plants, suggesting physical damage to the PSII. Sánchez-Moreiras et al. (2011) demonstrated that the reduction of Φ_{II} and F_v/F_m could be related to oxidative stress and necrotic processes, a finding that would be in accordance with the effects caused by *C. acaulis* EO, since *B. pilosa* leaves showed necrotic areas already at the lowest concentration tested (Fig. S1). In addition, a strong significant increment of regulated energy dissipation as heat (Φ_{NPQ}) and a significant reduction of non-regulated energy dissipation (Φ_{NPO}) was also observed after *C. acaulis* EO treatment. Since photosynthetic measures were taken on alive-leaves without many necrotic symptoms, increased levels of Φ_{NPQ} could be interpreted as a protection mechanism against stress, dissipating the excess energy in a regulated harmless way to overprotect the undamaged or less damaged leaves. In fact, Flores-Bavestrello et al. (2016) found that Φ_{NO} decreased under light-intensity situations when Φ_{NPQ} was effectively protecting the plant through the xanthophyll cycle.

Damages on the photosystem II can also be demonstrated through ionomic results after 48 h of EO exposition. *Bidens pilosa* plants experienced a significant reduction on the content of Mn, which is considered a crucial component of the oxygen-evolving complex (OEC) of PSII (Umena et al., 2011). The Mn_4Ca cluster of the PSII not only facilitates the oxidation of water molecules, providing the electrons necessary to drive the photosynthetic electron transport chain, but also protects the

Table 2
Metabolites statistically altered after *Carlina acaulis* essential oil treatment. Data were analysed through the Student's *t*–test ($p \leq 0.05$). A False Discovery Rate (FDR) was applied to the nominal *p*–values. Negative values of the *t*–stat value indicate a significant increase in the specific metabolite, and positive values indicate a significant reduction of the specific metabolite. N = 4.

Metabolites	*t*-stat	*p*-value	FDR	Class
5-Oxoproline	-3.9952	0.0071578	0.013743	**Amino acids**
β-Alanine	3.3393	0.015625	0.026786	
GABA	-3.9413	0.0076133	0.014331	
Isoleucine	-9.1008	9.89E-05	0.00055829	
L-Arginine	5.1156	0.0021877	0.0055268	
L-Asparagine	-4.1091	0.0062929	0.012329	
L-Valine	-5.8573	0.001094	0.0032819	
Ethanolamine	3.2755	0.016917	0.028493	**Amine**
Hydroxylamine	3.1998	0.018604	0.030328	
Tyramine	3.5236	0.012464	0.022158	
N-Methylethanolamine	-4.251	0.0053743	0.011042	
Alpha-Lactose	4.9349	0.0026172	0.0062812	**Sugars**
Allose	24.628	2.95E-07	1.41E-05	
Fructose	17.148	2.52E-06	2.69E-05	
Glucose	16.55	3.10E-06	2.98E-05	
Mannose	17.645	2.13E-06	2.55E-05	
Ribose	20.058	9.97E-07	1.91E-05	
Sucrose	1.91E-05	1.91E-05	0.0033187	
Tagatose	18.799	1.46E-06	2.34E-05	
Trehalose	2.34E-05	2.34E-05	0.0015154	
Xylose	-5.0616	0.002307	0.0056788	
Galactonic acid	-3.1983	0.018639	0.030328	**Sugar acids**
Ribonic acid	3.1329	0.020249	0.031867	
Threonic acid	-12.356	1.71E-05	0.00013245	
N-Acetyl-D-hexosamine	4.6144	0.0036367	0.0081192	**Amino sugars**
N-Acetylmannosamine	6.3224	0.00073164	0.0025085	
Aconitic acid	-10.975	3.40E-05	0.00023314	**Organic acids**
Citric acid	-12.26	1.79E-05	0.00013245	
Dehydroascorbic acid	10.282	4.94E-05	0.00031625	
Fumaric acid	21.058	7.47E-07	1.79E-05	
Glutaric acid	-6.1831	0.00082331	0.0027024	
Glycolic acid	7.9504	0.00021061	0.00096279	
Malic acid	9.1146	9.80E-05	0.00055829	
Malonic acid	3.4657	0.013375	0.023345	
Succinic acid	8.9456	0.00010893	0.00058098	
Shikimic acid	17.996	1.89E-06	2.55E-05	
1,4-Benzenedicarboxylic acid	5.6591	0.001308	0.0035877	**Miscellaneous**
3-Aminoisobutyric acid	4.4331	0.0044085	0.0096185	
4-Hydroxybenzoic acid	-4.8307	0.0029082	0.0066474	
Adenosine	6.7311	0.00052332	0.0020096	
Coumaric acid	-7.7643	0.0002401	0.0010477	
Isohexonic acid	-25.311	2.51E-07	1.41E-05	
Fructose 6-phosphate	-4.2457	0.0054058	0.011042	
Glycerol-3-Galactoside	-6.1534	0.00084451	0.0027024	
Gluconolactone	4.3952	0.0045923	0.009797	
Glucose 6-phosphate	-4.8312	0.0029067	0.0066474	
Quinic acid	21.107	7.37E-07	1.79E-05	
N-Acetyl-DL-serine	-13.971	8.38E-06	7.32E-05	
Hexitol	5.1312	0.0021545	0.0055268	**Sugar alcohols**
Iditol	3.6411	0.01082	0.019599	
Myo-inositol	6.364	0.00070655	0.0025085	

PSII complex from oxidative damage (Schmidt et al., 2016). For this reason, Mn deficiency alters water photolysis in PSII complexes, leading to a decreased number of electrons traversing the photosystem, which would induce a diminished photosynthetic efficiency. Consequently, Mn deficiency is directly correlated to the F_v/F_m ratio , which is reduced due to stress, as occurs after *C. acaulis* EO exposition. In addition, *Hordeum vulgare* L. leaves showed necrotic brown spots as a consequence of Mn deficiency (Schmidt et al., 2016), symptom also obtained on *B. pilosa* leaves after *C. acaulis* EO treatment (Fig. S1). The ionomic assay has also reported a reduced level of Ca after *C. acaulis* EO treatment. Since Ca is part of the Mn_4Ca cluster, Ca deficiency negatively affects the photosynthetic efficiency, owing to detrimental damages to the photosynthetic machinery of PSII.

Furthermore, significant high levels of K and low levels of Na were found after *C. acaulis* EO treatment. Potassium is well known to be the major solute involved in osmotic adjustment in some plant species (Ma et al., 2004; Moinuddin et al., 2005), helping to enhance the plant tolerance in drought-stress situations. It has been reported that under drought stress conditions, plant capacity to uptake K from the soil is reduced as water content decreases, suggesting that K accumulation in young leaves under drought stress is related to K internal translocation to improve the osmotic adjustment (Damon et al., 2011). Since our measurements were done on leaves that were not entirely wilted by EO treatment, the K accumulation on *B. pilosa* leaves could mean that the plants are trying to cope with water status alteration by increasing the osmotic adjustment through K mobilisation (Mahdid et al., 2021).

The untargeted metabolomic analysis pointed out that *C. acaulis* EO caused a significant generalised increment on the content of amino acid with osmoprotectants ability (i.e. alanine, GABA, isoleucine or valine, among others). Amino acid metabolism in plants plays an important role under abiotic stress conditions. Accumulation of different amino acids, considered osmoprotectants in plants, is well documented to be involved in stress tolerance. Similar effects were recently found by López-González et al. (2023) after citral and farnesene treatments,

Table 3
Result from ingenuity pathway analysis carried out with 'MetPa' on *Bidens pilosa* adult plants sprayed with *Carlina acaulis* essential oil for 48 h.

Pathway analysis	Total Cmpd	Hits	Raw p	FDR	Impact
Alanine aspartate and glutamate metabolism	22	8	0.000834	0.0022386	0.84892
Glycine serine and threonine metabolism	33	4	0.0065658	0.011547	0.39836
Starch and sucrose metabolism	22	5	9.38E-05	0.00044027	0.3404
Citrate cycle (TCA cycle)	20	5	1.11E-07	5.65E-06	0.29335
Glyoxylate and dicarboxylate metabolism	29	7	0.00072169	0.0020448	0.23247
Arginine and proline metabolism	34	3	0.0026869	0.0052704	0.18619
Arginine biosynthesis	18	6	0.0025712	0.0052453	0.16991
Tyrosine metabolism	16	2	2.19E-05	0.00013988	0.16892
Butanoate metabolism	17	4	1.98E-05	0.00013988	0.13636
Glutathione metabolism	26	4	3.92E-06	3.33E-05	0.13362
Sulfur metabolism	15	2	0.00026114	0.00083239	0.10774

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the uploaded data; Raw p: the original p value calculated from the enrichment analysis; FDR: false discovery rate. Only the pathways with impact higher than 0.1 were reported. The full list is available in Supplementary Table S1.

suggesting that plants were defending themselves from stress through increasing osmoprotectants production. High accumulation of amino acids, particularly the branched-chain amino acids (BCAAs), such as isoleucine and valine, have already been reported to be related to osmotic stress responses, mainly due to drought stress conditions (Joshi et al., 2010). High levels of BCAAs have been observed after *C. acaulis* EO exposition, corroborating that plants could be under drought stress. Huang and Jander (2017) also obtained BCAAs' accumulation in response to osmotic stress after 1 h of drought-stress exposition. They demonstrated that BCAA accumulation in response to stress occurs through abscissic acid-regulated protein degradation since this is a key regulator of osmotic stress responses. Our results could suggest that *C. acaulis* EO exposition causes an alteration of plant water status, inducing osmotic alterations and, as a consequence, accumulation of branched-chain amino acids.

Related to the reduced photosynthetic efficiency in EO-treated plants, it is also necessary to highlight the generalised decrease in sugar content observed after 48 h of EO treatment (sucrose, glucose, fructose, ribose, among others). Although soluble sugars usually accumulate under stress conditions inducing stress response signals to increase stress tolerance (Rosa et al., 2009), under several stress conditions, the sugar content can also decrease (Khan et al., 2020). Indeed, low sugar levels can be considered a plant response to abiotic stress conditions, such as drought, high temperature or salinity. Reduction in sugar content has already been observed in another study, in fact López-González et al. (2023) obtained a reduction in sucrose content after farnesene and citral treatments on *A. thaliana* seedlings, suggesting a relation with the reduction in photosynthetic efficiency, since it is the main photosynthetic product. This situation would be in accordance with our results since *C. acaulis* EO altered several chlorophyll *a* fluorescence parameters, suggesting damage in the photosynthetic machinery of *B. pilosa* plants, reducing the photosynthetic efficiency. Indeed, one of the most impacted pathways after *C. acaulis* EO treatment was the starch and sucrose metabolism.

5. Conclusions

The *C. acaulis* EO, composed mainly of the polyacetylene carlina oxide, has shown strong phytotoxic potential against the dicot weed *B. pilosa*, causing leaf necrosis, reduction in RWC and increment of DW/FW ratio, suggesting water status alteration. On the other hand, plants tried to counteract this stress through ion remobilisation and activation of the biosynthesis of primary metabolites with osmoprotectants activity. The effects caused by this EO suggest the induction of physical damage to the photosynthetic machinery since both Φ_{II} and F_v/F_m chlorophyll *a* fluorescence parameters decreased, and Φ_{NPQ} increased as a defence mechanism after EO treatment. The reduction in sugar, Mn, and Ca levels further confirmed those potential damages, suggesting an alteration in the correct functionality of the Mn_4Ca cluster of PSII. The findings of this work suggest that *C. acaulis* EO could be considered a promising phytotoxic product for weed management.

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CRediT authorship contribution statement

Sara Álvarez Rodríguez: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualisation. **Eleonora Spinozzi**: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualisation. **Adela M. Sánchez-Moreiras:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualisation. **David Lopez-Gonzalez:** Formal analysis, Investigation, Data curation. **Marta Ferrati**: Investigation, Data curation, Writing – review & editing, Visualisation. **Giorgio Lucchini:** Formal analysis, Validation, Data curation. **Filippo Maggi**: Conceptualization, Investigation, Data curation, Writing – review & editing, Visualisation. **Riccardo Petrelli**: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Visualization, Supervision. **Fabrizio Araniti:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.
Investigating the phytotoxic potential of Carlina acaulis essential oil against the weed Bidens pilosa through a physiological and metabolomic approach (Original data) (Mendeley Data)

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.117149.

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