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Phytochemical screening and phytotoxic activity of *Pinus ponderosa* (Dougl.) Lawson

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Abstract: Developing natural herbicides offers a potential solution to mitigate the drawbacks associated with synthetic pesticides used in an excessive quantity to safeguard agricultural crops. In this study, essential oils extracted via hydrodistillation from *Pinus ponderosa* needles were investigated for their chemical composition and phytotoxic activity. Gas chromatography with mass spectrometry detection (GC/MS) identified twenty-three constituents, constituting 93.87% of the total oil. The predominant components were oxygenated monoterpenes (64.66%), with α-pinene (37.78%), β-pinene (24.32%), and sesquiterpenes hydrocarbons, particularly germacrene-D (7.26%). The phytotoxic effects of *P. ponderosa* essential oil were tested on *Phalaris canariensis* L., *Trifolium campestre* Schreb., and *Sinapis arvensis* L. The essential oil exhibited a significant inhibitory effect on seed germination and seedling growth in a dose-dependent manner. A low concentration of essential oil reduced the germination and seedling growth of all tested weeds. Additionally, the essential oil treatment impacted malondialdehyde content and electrolyte leakage in the seedlings. These preliminary findings suggest that essential oils from forest trees, particularly *Pinus ponderosa*, could serve as an eco-friendly alternative to chemical herbicides. This approach may contribute to addressing the challenges associated with synthetic pesticides while promoting sustainable and environmentally friendly agricultural practices.

Keywords: essential oil; gas chromatography with mass spectrometry detection (GC/MS); malondialdehyde (MDA); phytotoxic effect; weeds

The challenges confronting crop production have intensified due to the ever-growing global demand and the substantial impact of climate change (Fahad et al. 2017). Among the myriad threats, pests stand out as the primary adversaries of agricultural crops, causing a significant reduction in crop yields ranging from 25% to 50% (Oerke et al. 2006). In an effort to protect crops, substantial amounts of synthetic pesticides are used. However, the excessive application of these pesticides in agricultural fields, water sources, urban areas, and the environment, aimed at eradicating harmful pests, has resulted in the exacerbation of issues such as pesticide resistance, increased pest resurgence, the emergence of toxico-

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logical implications for human health, and heightened environmental pollution (Bhattacharya et al. 2008; Hong et al. 2009).

Researchers are exploring a range of strategies to ensure global food security, concentrating on enhancing crops and substituting synthetic chemicals with safer, environmentally friendly alternatives (Khammassi et al. 2022). One such approach is the biological control of plant pests and diseases, which involves utilising plant secondary metabolites to bolster plant resistance against pests and diseases. Consequently, the evaluation of essential oils and plant extracts for their biological activities holds promise for discovering new molecules for effective pest control (Ismail et al. 2021).

Various phytochemicals extracted from diverse pine species have exhibited adverse effects on weeds, bacteria, fungi, and insects (Li et al. 2007; Ismail et al. 2021). The *Pinus* genus, comprising over 100 species and belonging to the *Pinaceae* family, is predominantly found in the Northern Hemisphere. Of particular significance is the ponderosa pine, a crucial resource for various products (Sohn et al. 2014). Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.), also known as western yellow pine, is among the most widely distributed and important pines in the western United States (Sohn et al. 2014). The essential oil derived from ponderosa pine demonstrates antimicrobial properties (Himejima et al. 1992; Krauze-Baranowska et al. 2002).

The essential oil of ponderosa pine has gained significant recognition in both domestic and international markets. For instance, Krauze-Baranowska et al. (2002) emphasised its potent antifungal properties, exhibiting superior activity against *Fusarium culmorum*, *Fusarium solani*, and *Fusarium poae* when compared to oils derived from *Pinus resinosa* or *Pinus strobes*. It is crucial to note that the production of essential oils is influenced by various factors, including genetic background and geographical origin (Baranowska et al. 2002).

Although the chemical composition of Tunisian *Pinus ponderosa* essential oil has been previously documented, there is currently no available information regarding its herbicidal effects. Consequently, this study has two primary objectives. Firstly, we aim to investigate the chemical composition of the essential oil extracted from *P. ponderosa* specimens grown in Tunisia. Subsequently, in the second phase, we intend to assess the herbicidal activity of the obtained essential oil.

MATERIAL AND METHODS

Plant material. The needles of *Pinus ponderosa* were collected from the arboreta of the National Institute for Research in Rural Engineering, Water and Forests. Five batches of needles were taken from different trees and mixed to have a homogeneous sample. The plant was identified by Dr Lamia Hamrouni, National Institute for Research in Rural Engineering, Water and Forests, Tunisia, and a sample (PR-1110) was submitted to the herbarium division of the Institute.

Isolation of the essential oils. Three replications of 100 g of air-dried needles were submitted to hydro-distillation for three hours with 500 mL distilled water using a Clevenger-type apparatus (ISOLAB, Germany). The volatile oils were collected and dried over anhydrous sodium sulphate and stored in sealed glass brown vials in a refrigerator at 4 °C. Yield based on dried weight of the sample was calculated (*w*/*w* %) (Souihi et al. 2020a).

Gas chromatography analysis with mass spectrometry detection. Analysis of essential oil (EO) was carried out using a Hewlett Packard 5890 II GC (Agilent Technologies Inc., USA), equipped with a capillary column HP-5 MS (30 m × 0.25 mm, film thickness 0.25 µm) and a mass selective detector HP5972. The oven temperature was kept at 50 °C for 1 min, then programmed from 50 °C to 250 °C at 5 °C·min^{-1} and subsequently held isothermal for 4 min. The carrier gas was helium at a flow rate of 1.2 mL·min^{-1}. In GC/MS detection, an electron ionisation system with a scan time of 1.5 s and mass range 40–300 amu with ionisation energy of 70 eV was used. At 250 °C, the injector was set and transfer line temperatures at 280 °C. In the splitless mode, samples were diluted in hexane (1/10, *v*/*v*), and then 1 µL of the solution was injected. The identification of oil components was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library or with standard compounds), and expressed by comparing their retention index with those of authentic compounds or based on the results published in the literature (Ismail et al. 2014; Souihi et al. 2020b). Another confirmation was done based on data generated from a series of n-alkanes retention indices (C9–C28) on the HP-5 MS capillary column.

Seed germination experiment (Petri dish experiment). The phytotoxic effect of EO was determined by a Petri dish method with slight modifications (Ayed et al. 2023). Essential oil was decomposed in Tween 80 (1%) with different concentrations (1–4 µL·mL^{-1}).

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Mature seeds of weeds of *Sinapis arvensis* L. *Phalaris canariensis* L. and *Trifolium campestre* were collected from parent plants growing in fields in July. The seeds were disinfected with NaCl (0.5%) for 10 min and rinsed multiple times with distilled water. Distilled water and 1% Tween 80 were used as negative control. Two pieces of sterilised filter paper were placed in a sterilised Petri dish with a diameter of 9 cm. Essential oil solution (5 mL) was added to the test group. 30 seeds of weed seeds were evenly spread on the filter paper, then the Petri dish was covered immediately, and placed in an artificial climate chamber (25 ± 2 °C) for culture. The standard for seed germination is that the radical or hypocotyl can break through the seed coat by 1–2 mm. The number of plant seeds germinating was observed and recorded every day for seven consecutive days (until no more germination for 3 consecutive days). The shoot length and root length were measured with a digital display Vernier calliper on the 7th day. Each treatment was repeated three times. The final germination rate was calculated according to the following Equation (1):

$$Germination\ rate = \frac{A}{A_0} \times 100\% \quad (1)$$

where:

A – total number of germinated seeds;

A_0 – total number of tested seeds.

Post-emergence assays. The further phytotoxic effect of EO was assessed with pot assay according to the previous experiment with slight modifications (Kapoor et al. 2019). A small pot (height 7.5 cm and width 6.0 cm) was filled with 185 g of sterilised soil, 30 sterilised seeds were evenly sown in the pot, and 10 mL of EO and control solutions were applied to water the pots for two weeks, respectively. After the emergence of the seedlings, a sprayer was used to spray 5 mL of EO and control solutions on each pot for one week, respectively. This was repeated for each treatment group six times. After spraying for a week, malondialdehyde (MDA) content and electrolyte leakage (*EL*) were determined.

Determination of malondialdehyde content. Malondialdehyde (MDA) content was assessed using a triclosan abrasion according to the method of Heath and Packer (1968). Frech leaves (200 g) were extracted with trichloroacetic acid (TCA 0.1%, *w*/*v*) and centrifugated at 10.000 tours for 10 min. Supernatant (1 mL) was added to 4 mL of 0.5% thiobarbituric acid in 20% TCA. After 30 min of incubation at 90 °C followed by cooling over ice and centrifugation, the absorbance of supernatant was determined at 532 nm and correlated for non-specific absorbance at 600 nm. MDA content was expressed as nmol·g^{-1} fresh weight using the extinction coefficient of 155 mm × 1 cm^{-1} (Khammassi et al. 2023; Khedhri et al. 2023).

MDA amount was determined using the extinction coefficient of 155 mm × 1 cm^{-1}. The MDA amount was expressed as nmol·g^{-1} fresh weight.

Relative electrolyte leakage (*REL*). *REL* was determined in treated leaves and calculated following Equation (2):

$$REL\ (\%) = \frac{C_1}{C_2} \times 100 \quad (2)$$

where:

C_1 – conductivity measured after the immersion of leaves in glass boxes containing distilled water for 60 min;

C_2 – conductivity of leaves measured after 30 min in boiling water.

Statistical analysis. Data analysis of seed germination and seedling growth was conducted using one-way analysis of variance (ANOVA) in the SPSS software package (Version 23.0, 2015) for data related to seed germination and seedling growth. Differences between means were assessed using the Student-Newman-Keuls test and means with *P*-values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Essential oil. The hydro-distillation of dried *Pinus ponderosa* needles yielded a yellowish essential oil (0.90%, *w*/*w*). The oil yields observed in this study were more significant compared to those reported in Poland, which were 0.30% (Krauze-Baranowska et al. 2002), and 0.60% in Japan (Kurose et al. 2007). GC/MS analysis identified a total of 23 constituents, constituting 93.87% of the total oil. The components were classified into three subclasses, with hydrocarbonated monoterpenes contributing the most percentage (64.66%), followed by hydrocarbonated sesquiterpenes (25.72%) and the oxygenated monoterpenes represented only 2.98%. The compounds, listed in order of elution on the apolar HP-5 MS column, along with quantitative data, are presented in Table 1.

The chemical composition of needles EO of *P. ponderosa* contained α-pinene (37.78%), β-pinene

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Table 1. Chemical composition of *Pinus ponderosa* essential oil

No.	Retention index	Compounds	*Pinus ponderosa* (%)
1	939	α-pinene	37.78
2	954	camphene	1.59
3	976	β-pinene	24.32
4	991	β-myrcene	0.66
5	1 007	α-phellandrene	2.32
6	1 021	1,8-cineole	0.96
7	1 037	(Z)-β-ocimene	0.31
8	1 235	methyl thymol ether	0.13
9	1 332	δ-elemene	0.52
10	1 354	α-cububene	1.02
11	1 376	α-copaene	1.82
12	1 388	β-bourbonene	0.10
13	1 398	longifolene	2.03
14	1 419	β-cedrene	0.64
15	1 420	(Z)-caryophyllene	0.97
16	1 441	aromadendrene	0.88
17	1 477	δ-gurjunene	1.60
18	1 478	germacrene-D	7.26
19	1 484	α-amorphene	1.25
20	1 491	germacrene-A	0.64
21	1 499	α-murrolene	1.10
22	1 524	δ-cadinene	5.89
23	1 576	caryophyllene oxide	0.51
Total identification			93.87
Monoterpenes hydrocarbons			64.66
Oxygenated monoterpenes			2.98
Sesquiterpenes hydrocarbons			25.72
Oxygenated sesquiterpenes			0.51

(24.32%) and germacrene-D (7.26%) as the most abundant compounds (Figure 1).

The EO of *P. ponderosa* has been previously examined in various regions, and our findings align with existing data. Previous studies have indicated that β-pinene (0.1–38.2%) and α-pinene (0.2–51.8%) are the predominant components in essential oils from Japan (Kurose et al. 2007). However, the *P. ponderosa* EO from the western United States reported varying ranges for α-pinene (18% to 69%) and β-pinene (2% to 57%) (Thoss, Byers 2006). Similarly, the essential oil of *P. ponderosa* from Poland contained α-pinene (18.4–67.2%) and β-pinene (3.3–50.8%) (Krauze-Baranowska et al. 2002). To maintain the standardisation of essential oil composition, it is crucial to ensure several key aspects. This includes the implementation of ethical harvesting practices to prevent overexploitation of plant species, rotational harvesting, adherence to seasonal cycles, which can help maintain biodiversity, cultivate plants sustainably, ensure the conservation of wild populations, and optimise the delivery method of simple extraction conditions (Souiy 2024).

Seed germination experiment. In the seed germination experiment, the herbicidal effect of *P. ponderosa* EO was assessed on seed germination and seedling growth. Table 2 demonstrates that the essential oil significantly inhibited the germination and seedling growth of the tested weeds in a dose-dependent manner, exhibiting similar effects on dicots (*S. arvensis* and *T. campestre*) and monocots (*P. canariensis*), which are common weeds in Tunisia.

Our results confirm that germination and seedling growth were reduced under essential oil treatment,

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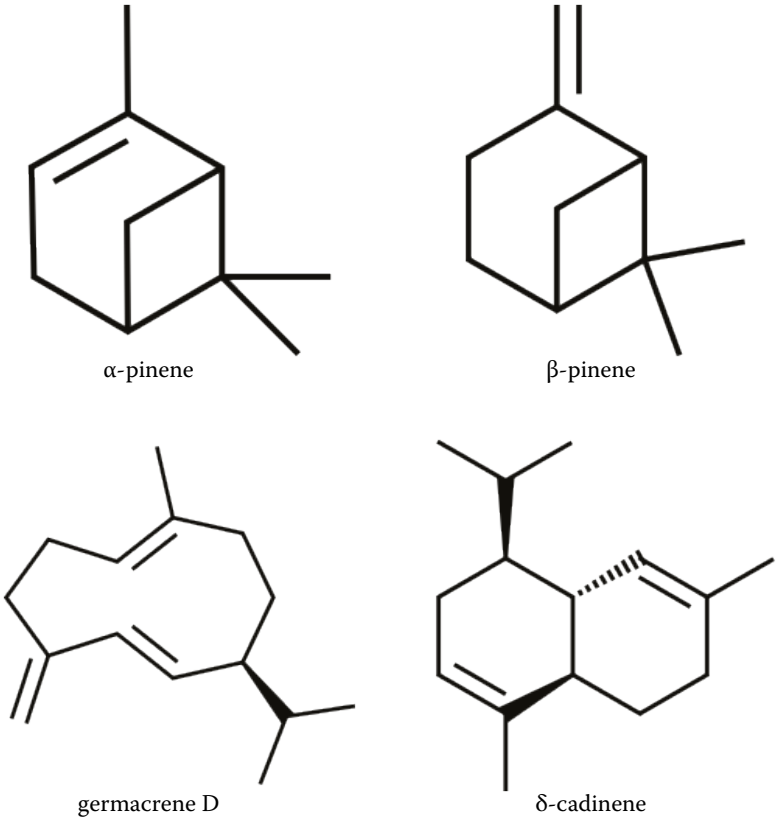

Figure 1. Major components of *Pinus ponderosa* essential oil

and all tested weeds exhibited sensitivity to the essential oil at both germination and development stages (Table 2).

Seed germination of *S. arvensis* was half reduced under the application of 1 μL·mL^{-1} EO and completely inhibited under 4 μL·mL^{-1} essential oil. For the rest

Table 2. Herbicidal activity of *Pinus ponderosa* essential oil against weeds

Weeds	Dose (μL·mL^{-1})	Germination (%)	Seedling growth (mm)	
			aerial parts	roots
Sinapis arvensis	0	96.66 ± 5.77^{e}	14.40 ± 0.96^{e}	12.53 ± 1.50^{e}
	1	55.33 ± 5.77^{d}	8.53 ± 0.75^{b}	8.40 ± 0.79^{d}
	2	36.66 ± 5.77^{c}	6.80 ± 0.75^{c}	4.40 ± 0.52^{c}
	3	13.33 ± 5.77^{b}	3.34 ± 0.92^{b}	1.86 ± 0.15^{b}
	4	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Trifolium campestre	0	86.66 ± 5.77^{e}	12.06 ± 0.90^{d}	15.50 ± 1.50^{e}
	1	66.66 ± 2.88^{d}	10.73 ± 1.25^{d}	8.43 ± 0.58^{d}
	2	36.66 ± 10.00^{c}	6.233 ± 1.16^{c}	5.93 ± 0.20^{c}
	3	20.00 ± 10.00^{b}	2.73 ± 0.25^{b}	2.36 ± 0.55^{b}
	4	6.66 ± 5.70^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Phalaris canariensis	0	93.33 ± 5.77^{c}	12.30 ± 0.65^{e}	13.66 ± 0.57^{e}
	1	65.00 ± 5.00^{c}	8.53 ± 0.50^{d}	10.06 ± 0.11^{d}
	2	38.33 ± 16.07^{b}	6.76 ± 0.68^{c}	7.43 ± 0.51^{c}
	3	15.00 ± 5.00^{a}	2.73 ± 0.25^{b}	3.80 ± 0.43^{b}
	4	13.33 ± 6.30^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

$^{a–e}$Statistical differences by the Student-Newman-Keuls test ($P \leq 0.05$)

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of the weeds, *T. campestre* and *P. canariensis*, 1 μL·mL^{-1} essential oil has reduced almost 40% of seed germination. The use of 4 μL·mL^{-1} essential oil showed 6.66% germination of *T. campestre* and 13.33% germination of *P. canariensis* seeds, with a total inhibition of seedling growth for both seeds (Figure 2).

To the best of our knowledge, this study represents the first investigation into the phytotoxic effects of *P. ponderosa* essential oils. In previous reports, we have demonstrated the phytotoxic potential of essential oils from various species belonging to different families, including *Pinaceae*, *Cupressaceae*, and *Anacardiaceae* (Amri et al. 2011, 2012). Recently, we showed that *P pinea*, *P. radiata*, and *P. patula* exhibit inhibitory effects on the germination and seedling growth of *Sinapis arvensis*, *Lolium rigidum*, and *Raphanus raphanistrum* (Ismail et al. 2021). This inhibitory effect is commonly attributed to the main components, and in our present study, *P. ponderosa* oil, rich in oxygenated monoterpenes, especially α-pinene and β-pinene, is known for its phytotoxic effects (Amri et al. 2012).

Knowing that essential oils especially terpenes had been identified as the major sources of secondary

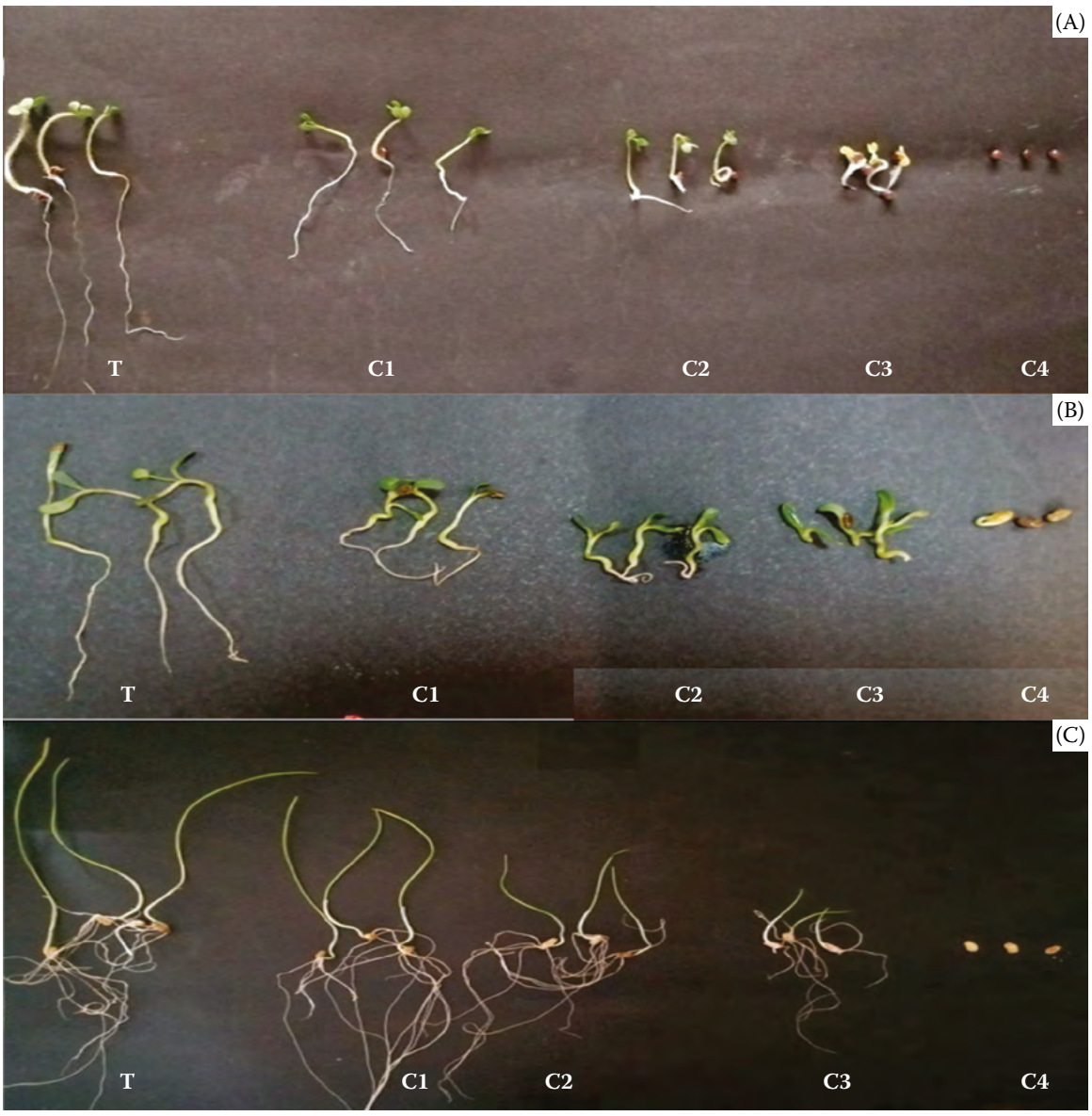

Figure 2. The effect of essential oil (EO) doses on seedling growth of (A) *Sinapis arvensis*, (B) *Trifolium campestre*, and (C) *Phalaris canariensis*

T = 0 μL·mL^{-1}; C1 = 1 μL·mL^{-1}; C2 =2 μL·mL^{-1}; C3 = 3 μL·mL^{-1}; C4 =4 μL·mL^{-1}

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aerosols in the atmosphere, which can come from biogenic emissions and anthropogenic emissions (Sahu, Sahu 2012), we are trying to minimise the doses used.

Although the exact inhibitory action mechanism of EO on weed seed germination remains unclear, we further investigated the effect of the EO on plant growth.

Post-emergence assays: MDA content and *REL*. The malondialdehyde (MDA) content reflects the extent of membrane peroxidation in plant cells (Figure 3A). After essential oil spraying, visible lesions, chlorosis, necrosis of leaves, and complete wilting of all tested weeds were observed. This outcome underscores the phytotoxic potential of *P. ponderosa* EO and elucidates its mechanism of action. The membrane integrity of the tested plants was assessed by measuring relative electrolyte leakage, which is determined by the increase in the bathing medium conductivity.

As depicted in Figure 3B, different concentrations of *P. ponderosa* essential oils had varying effects on electrolyte leakage in different weeds.

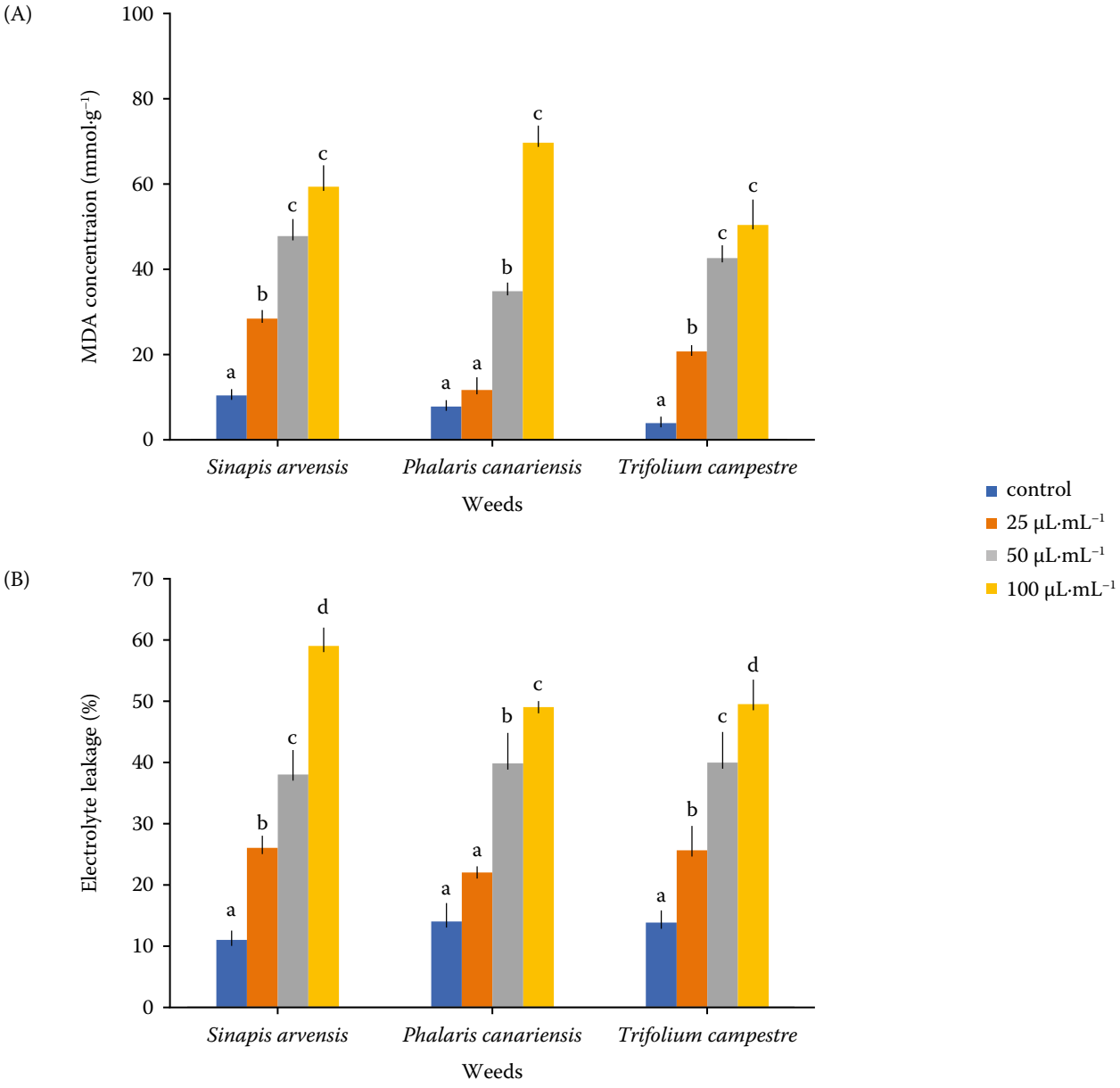

Figure 3. The effect of different concentrations of *Pinus ponderosa* essential oil on (A) malondialdehyde (MDA) concentration and (B) electrolyte leakage

a–d – statistical differences by the Student-Newman-Keuls test ($P \leq 0.05$)

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According to the statistical analysis, electrolyte leakage was highly pronounced, indicating a varied phytotoxic effect of the essential oil based on the dose and the tested plant. The application of 100 $\mu L \cdot mL^{-1}$ of essential oil induced the most pronounced effects on all plants: 59.8%, 50.3%, and 48.5% for *S. arvensis*, *T. campestre*, and *P. canariensis*, respectively.

While the exact modes of action of essential oils applied by spraying remain understudied, numerous results have indicated their ability to inhibit weed growth after post-emergence application (Kong et al. 2021). Other studies have shown that the post-emergence application of herbicides is as effective as two manual weedings for better weed control, higher crop yields, and increased benefits (Kumar, Sarkar 2020). *P. ponderosa*, among its physiological effects, caused a loss of membrane integrity essential for vital functions and various roles, leading to excessive electrolyte leakage. The chemical composition of *P. ponderosa* essential oil, particularly the hydrocarbonated monoterpenes, α-pinene and β-pinene, known for their phytotoxic effects, contributed to these observed activities (Amri et al. 2023). Previous studies demonstrate that the consistent rise in MDA content and *EL* signifies an escalation in the peroxidation degree of the plant cell membrane, indicative of progressively intensified damage to the cell membrane structure (Varona et al. 2009; Khammassi et al. 2023).

CONCLUSION

In conclusion, the EO of *P. ponderosa*, obtained by hydro-distillation, were primarily composed of monoterpene hydrocarbons, with α-pinene and β-pinene dominating the chemical profile. In agronomy, *P. ponderosa* EO displayed a significant phytotoxic effect against the germination and seedling growth of tested weeds, representing the first report on the herbicidal activity of *P. ponderosa* essential oil for these three weeds. These findings suggest that incorporating such bio-molecules in herbicidal formulations could contribute to the development of natural herbicides for sustainable agriculture, potentially reducing the reliance on synthetic pesticides and addressing issues such as resistance and environmental pollution.

In this case essential oils poses challenges for their application, as they can evaporate quickly, reducing their efficacy. Recent advances in encapsulation techniques, such as the use of dendrimers or β-cyclodextrin and the development of nanoemulsions has been explored to improve the stability and control release of essential oils. Our preliminary results propose forest tree essential oils, especially from pine species, as an ecological alternative to chemical herbicides.

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