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Unveiling the efficacy of pre-emergent application of young *Eucalyptus globulus* leaves as a weed control strategy: bridging macroscopic effects and cellular responses

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Abstract

Allelopathy, the inhibition of neighbouring plant growth by certain plants, can be particularly useful if applied in a targeted way for weed management. So, this study aimed to assess and characterize the herbicidal activity of fresh and dried leaves from young *Eucalyptus globulus* Labill. trees applied as a soil amendment. For this, fresh and dried leaves (FL and DL, respectively) were incorporated into the soil at different concentrations (0, 1, 5, and 10% w/w), where *Portulaca oleracea* L. seeds were sown. After 5 weeks of exposure, results revealed that the soil incorporation of DL at 10% (w/w) presented the strongest herbicidal properties, inhibiting seed germination by 63% and inducing the loss of cell viability. To unravel the possible mode of action and the main targets at both cellular and subcellular levels, an *in vitro* experiment was performed.

Purslane seeds were sown in a nutritive medium containing different dilutions of an aqueous extract prepared with dried eucalyptus leaves. After 5 days of exposure, germinated seedlings were processed for transmission electron microscopy and histological analyses as well as for reactive oxygen species (ROS) *in vivo* detection by confocal laser scanning microscopy. Results revealed that the allelochemical release from DL induced ROS overproduction, resulting in the loss of cell integrity and organization, which was characterized by damage to several cellular sub-structures, along with enhanced accumulation of lipid droplets. Overall, the incorporation of DL into the soil can represent a sustainable alternative to reduce synthetic herbicide application and subsequent environmental contamination.

Keywords: Sustainable agriculture; bioherbicide; post-fire management; allelopathy; phytotoxic effects; weed management.

1. INTRODUCTION

To increase crop yield and quality in an ever-changing environment, farmers are increasingly dependent on synthetic pesticide applications to eliminate pests and pathogens, such as bacteria, fungi, molluscs insects, rodents, nematodes, and weeds (Aktar et al., 2009;Dar et al., 2020). In fact, according to recent data, pesticide application has been increasing since 1990, having surpassed, on average, the mark of 2.5 kg ha^{-1} at the global level (*http://www.fao.org/faostat/en/#data/EP/visualize*).

Of all the existing pesticides, herbicides are the most applied in the world, followed by insecticides and fungicides (Hernandez et al., 2013), which account for over 60% of all the pesticides used in agriculture (Kaur, 2019). Indeed, the herbicide market is a high-volume, growing business worldwide, with herbicide sales increasing nearly 14% compared to the previous year (https://www.spglobal.com/commodityinsights/en/ci/products/crop-science-pesticides.html). However, despite its great effectiveness in eliminating weeds, major

drawbacks have arisen from its continuous and cumulative application, such as resistance acquisition by several weed species (Benchaa et al., 2018) and contamination of the environmental spheres (air, water, and soil) with potential effects on different living organisms (Hernandez et al., 2013;Geetha, 2019;Soares et al., 2019b;Shahena et al., 2020). In this sense, new ecological solutions that can be used in the short term to prevent weed proliferation, allowing for more sustainable and affordable weed management, must be found. This has motivated the scientific community in the last decade to find environmentally friendly alternatives that could be implemented in modern agriculture practices to reduce the application of synthetic herbicides (Hammermeister, 2016;Balaure et al., 2017;Pinto et al., 2021;Zhang et al., 2022). These strategies can range from the application of organic mulches (Abouziena and Radwan, 2015), nanoparticles encapsulating synthetic herbicides (Sousa et al., 2018) or plant-based bioherbicides (Lins et al., 2019;Pinto et al., 2021). Despite the multiple approaches proposed by several authors, most scientists agree that the employment of an integrated weed management, through the combination of preventive, mechanical, cultural, biological, and chemical practices, is crucial to avoid weed resistance and reduce detrimental environmental impacts (Ofosu et al., 2023).

Throughout evolution, plants have developed an effective specialized metabolism that enables them to cope with challenges not directly involved with growth and reproduction. These challenges can range from herbivore attacks, intra- or inter-specific competition to phytopathogenic infections (Korkina, 2007). One of the most remarkable examples of this feature of the plant kingdom is the allelopathy phenomenon (Rice, 1984). Through the release of allelochemicals, some plant species can prevent the growth of surrounding plants, thus avoiding competition for natural resources, such as light, water, and nutrients (Rice, 1984). Areas dominated by eucalyptus trees are a classic example of the potency of allelopathy, as other plants are rarely found in those plantations. Therefore, the natural allelopathic properties of eucalyptus, especially

attributed to their leaves, could be used in a targeted way to inhibit the growth of undesirable plant species, such as weeds.

At the same time, the use of eucalyptus' specialized metabolism for bioherbicide production may represent a new way of controlling this species' dispersion outside managed plantations, thus decreasing fire risk, by reducing the fuel supplied by the trees. Indeed, the favourable wood characteristics of eucalyptus trees, mainly *Eucalyptus globulus* Labill., for pulpwood production, along with their great adaptability to different environmental conditions, have contributed to its wide geographic distribution outside its native range, southeast Australia (Catry et al., 2015). However, in a monoculture regime, *E. globulus* creates, around and between them, a drier and warmer environment that gives rise to fire-prone scenarios (Louro, 2016). Moreover, due to its fire-adaptive reproduction traits, *E. globulus* displays a unique vegetative regeneration strategy, resprouting, which allows a rapid and efficient plant recovery after wildfire occurrence (Catry et al., 2013). These eucalyptus characteristics confer them a competitive advantage over other species, and most, if not all, affected eucalyptus trees are expected to survive and regrow more quickly in a post-fire environment (ICNF, 2006), complicating post-fire management efforts carried out by rural inhabitants. Therefore, taking advantage of the allelopathic properties of post-fire regenerated eucalyptus trees to develop an eco-friendly and easily obtainable herbicide could be a way of valuing their foliar biomass and would benefit rural-urban inhabitants.

The biocidal potential of the leaves from young *E. globulus* trees has already been explored in a previous study of our research group (Pinto et al., 2021), where aqueous extracts prepared with fresh or dried leaves were foliar-applied to *Portulaca oleracea* L. (purslane) seedlings. The study showed that the dried leaf extract had strong herbicidal activity against purslane seedlings by inducing severe redox disorders in the roots (Pinto et al., 2021). However, the pre-emergent biocidal potential of eucalyptus leaf biomass regenerated after wildfire occurrence is still unclear. Until now, only two works have tested the herbicidal potential of incorporating eucalyptus leaf biomass into the soil (El-

Rokiek et al., 2011;Puig et al., 2013). Although in these studies different types of eucalyptus leaves were tested - El-Rokiek et al. (2011) incorporated dried and powdered leaves into the soil, while Puig et al. (2013) mixed fresh leaf fragments into the soil - both concluded that leaf residues from adult *E. globulus* trees can be used in weed control (El-Rokiek et al., 2011;Puig et al., 2013). Still, the mode-of-action and cellular targets of eucalyptus-based herbicides remain to be uncovered.

In this sense, the present work aimed to evaluate the pre-emergent herbicidal potential of the leaf biomass of young trees of *E. globulus* regenerated after a wildfire occurrence in *P. oleracea* and to understand the cellular and subcellular repercussions of the most effective dose of the bioherbicide in the physiological processes involved in weed control. By employing a multidisciplinary approach, it was concluded that the incorporation of dried leaves into the soil in a pre-emergence context exhibited potent herbicidal activity. This activity was attributed to the induction of strong ROS bursts, which caused significant alterations in cell structure and ultrastructure.

2. MATERIALS & METHODS

2.1. Collection and processing of the eucalyptus leaves

Leaf biomass from post-fire regenerated *E. globulus* trees with about six months old was collected in a recently burnt forest area of Porto, Portugal (GPS coordinates: 41.197288, -8.534506). The collected leaves were detached from the stems and, after oven-drying part of the leaves (at 60 ºC until reaching a constant weight), dried (DL) and fresh leaves (FL) were reduced to small fragments, to promote the release of allelochemicals, and directly used as a pre-emergent bioherbicide. Leaf processing also allowed the determination of the water content of eucalyptus leaves, which is 59 ± 0.6% (w/w).

2.2. Plant species

Seeds of *Portulaca oleracea* L. (purslane) were acquired from a local supplier and used as a weed plant model. Seeds were surface disinfected with 70% (v/v) ethanol, followed

by 20% (v/v) commercial bleach (5% active chlorine) for 10 min each, and successively washed with deionised water. Visually damaged seeds were discarded before sowing.

2.3. Evaluation of the pre-emergent herbicidal potential of the young eucalyptus leaves against purslane plants

2.3.1. Experimental design and growth conditions

To assess the herbicidal potential of young *E. globulus* leaves in a pre-emergent context, FL or DL were incorporated, individually, at different percentages [1, 5, and 10 % (w/w)] into an artificial soil (OECD, 2006). This soil consisted of 70% (w/w) sand, 20% (w/w) kaolin, and 10% (w/w) peat, with pH KCl 6.0 ± 0.5 that was prepared following the Organisation for Economic Cooperation and Development (OECD) guidelines (OECD, 2006). Eucalyptus leaves were incorporated into this artificial soil at different percentages for a total of 150 $g_{dry\ weight}$ per replicate (defined as each pot). For the highest concentration tested, 15 $g_{dry\ weight}$ of DL were incorporated into 135 $g_{dry\ weight}$ of soil and 9 $g_{dry\ weight}$ of DL were incorporated into 141 $g_{dry\ weight}$ of soil. For the control (CTL) situation, only standard artificial soil was used. At the beginning of each assay, the maximum soil water holding capacity (WHC_{max}), previously determined (ISO, 2005), was adjusted to 60%. Then, mixtures of leaves and soil were left to stabilize for two weeks, at room temperature, shaking three times a week, to facilitate the release of allelochemicals from the leaves into the soil. As a positive CTL, s-metolachlor (EFICA™ 960EC; 960 g L^{-1} s-metolachlor), a synthetic pre- and post-emergent herbicide, was considered. Based on the manufacturer's recommendations, an application rate of 1 L ha^{-1} was selected. According to the pot area (0.011 m^2) and the concentration of s-metolachlor, the conversion of this rate to the amount of the active ingredient per kg of soil equals 7.04 mg kg^{-1}. In this case, the adequate water volume for the WHC_{max} adjustment was used as a carrier to obtain the recommended concentration of s-metolachlor in the soil. Each situation comprised 4 biological replicates in which 20 seeds of *P. oleracea* were sown.

The assays were carried out in plastic pots, which had a cotton rope inserted at the bottom that was responsible for establishing the communication between the pot and a cup where the irrigation solution was placed. At the beginning of the assay, cups were irrigated with 100 mL of Hoagland solution (Taiz et al., 2015) to ensure nutrient availability, and then in the following weeks, irrigation was performed with deionised water. Experiments were conducted in a growth chamber with controlled conditions [temperature: 25 ºC; photoperiod: 16 h/8 h light/dark; photosynthetically active radiation (PAR): 120 μmol m^{-2} s^{-1}]. The number of germinated seeds and macroscopic phytotoxicity symptoms were monitored twice a week. At the end of the growth period (5 weeks), purslane plants were collected and used for biometric evaluation and cell viability analysis.

2.3.2. Biometric evaluation

After separating purslane plants into roots and shoots, the length and fresh weight of both organs were evaluated. The fresh biomass of each replicate was measured using a digital scale. Shoot length was measured from the apex to the shoot-root transition.

2.3.3. Histochemical detection of cell death

The cell death was evaluated according to Romero-Puertas et al. (2004), with brief modifications. For this purpose, excised leaves were infiltrated with 0.25% (w/v) Evans Blue through the petioles for 5 h. For each situation, leaves from three purslane plants were used. At the end of the incubation period, leaves were transferred to boiling 96% (v/v) ethanol for removing the photosynthetic pigments and to develop the blue precipitates. The presence of blue spots on the leaf surface is indicative of cell death. The results were photographically recorded.

2.4. Assessment of the phytotoxic effects induced by the pre-emergent application of young eucalyptus leaves

2.4.1. Experimental design and growth conditions

To unravel the cellular targets of the allelochemicals released by DL, an *in vitro* assay using an aqueous extract prepared with DL (250 $g_{dry\ weight}\ L^{-1}$) was performed. The extract was prepared according to Pinto et al. (2022) and, then, filtered through 0.2 µm nitrocellulose filters. Purslane seeds, disinfected as previously referred, were placed in Petri dishes containing half-strength Murashige and Skoog (1962) medium, 1.5% (w/v) agar, and the aqueous extract at 250 $g_{dry\ weight}\ L^{-1}$. A lower concentration of the aqueous extract was also included (31.25 $g_{dry\ weight}\ L^{-1}$) to get a better insight into the potential mode-of-action of eucalyptus allelochemicals in purslane cells. The control situation was prepared by replacing the aqueous extract with deionised water. For each experimental situation, four replicates were considered. Then, seeds were transferred to a growth chamber (temperature: 25 ºC; photoperiod: 16 h/8 h light/dark; PAR: 120 µmol $m^{-2}\ s^{-1}$) and left for germination for 7 days. At the end of the incubation period, the seedlings were separated into radicles and primordial shoots and processed to be analysed by optical microscopy (OM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM).

2.4.2. Histological analysis by OM

Shoots and radicles of treated and untreated purslane seedlings were firstly fixed in a 5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde mixture (2.5% glutaraldehyde: 2% paraformaldehyde) and then post-fixed in 1.5 mL of 1% (w/v) osmium tetroxide (OsO_4), which was prepared in 0.1 M sodium cacodylate buffer (pH 7.2) (Soares et al., 2020). After that, samples were dehydrated using increasing concentrations of ethanol (70, 80, 90, and 100% v/v), followed by EMBed-812 embedding. Semi-thin sections were obtained using an ultramicrotome (RMC Ultramicrotome PowerTome PT XL, USA). Then, semithin sections (≈1 µm) were stained with 1% (w/v) toluidine blue and observed

in a Zeiss Axio Imager AZ microscope with differential interference contrast optics (Zeiss, Göttingen, Germany).

2.4.3. Ultrastructural analysis by TEM

Both organs of purslane seedlings were processed for TEM as described by Soares et al. (2020). Sample fixation, post-fixation, dehydration, and embedding were performed as described in 2.4.2. Afterwards, ultra-thin sections were performed using an ultramicrotome. To assure random pictures, from control and treated samples, ultra-thin sections were obtained from different blocks, mounted in grids, and contrasted with uranyl acetate and lead citrate. Lastly, ultra-thin sections were observed under a Zeiss EM C10 TEM (Zeiss, Göttingen, Germany).

2.4.4. ROS *in vivo* detection by confocal laser scanning microscopy

The analysis of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) in cells of purslane radicles and cotyledons was performed based on the method of Spormann et al. (2022), by using fluorescent probes - 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) and dihydroethidium (DHE, Sigma-Aldrich)-, respectively. In brief, sections of both organs of the seedlings exposed to different concentrations (0, 31.25, and 250 $g_{dry\ weight}\ L^{-1}$) of the aqueous extract were incubated with 25 µM DCF-DA and DHE solutions, both prepared in 10 mM Tris-HCl buffer (pH 7.5) at 37 ºC for 45 min. Additionally, plant sections from all treatments were incubated with Tris-HCl buffer and considered as a negative control in the analysis. Upon the incubation period, the plant material was washed with 10 mM Tris-HCl buffer (pH 7.5) to remove the excess of fluorescent probes and samples were mounted in Tris-HCl buffer between a slide and a coverslip. The analysis and image acquisition was carried out using a Laser Point Scanning Confocal System (Leica TCS SP5-Leica Microsystems, Germany), detecting the fluorescent probe by monitoring the excitation and emission wavelengths of 480 and 530 nm, respectively. Image processing was performed using the ImageJ-Fiji program (Schindelin et al., 2012).

2.5. Statistical analysis

Results were expressed as mean ± standard deviation (SD) and all experimental situations comprised, at least, three replicates (n ≥ 3). For determining significant differences between treatments, a two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, whenever $p \leq 0.05$. The statistical analysis was performed using GraphPad Prism® 7.0 software (GraphPad Software, USA).

To test significant differences through time in the number of germinated seeds between treatments, a repeated-measures ANOVA was employed, defining the factors "within subjects" and "between subjects" as "weeks" and "applied concentration of FL/DL". When significant differences were found ($p \leq 0.05$), by assuming the correction of Greenhouse-Geisser, a one-way ANOVA, followed by Dunnett's post-hoc test, was performed for each factor. This analysis was performed in SPSS Statistics 22 (IBM SPSS®, USA).

3. RESULTS

3.1. Pre-emergent biocidal potential of young eucalyptus leaves against purslane plants

3.1.1. Number of germinated seeds over time

The number of germinated seeds was not affected by the addition and mixture of FL into the soil, in any of the tested concentrations in comparison to the negative (0% w/w) and positive (s-metolachlor) CTL situations (**Fig. 1A**). Regarding the incorporation of DL into the soil, the highest concentration inhibited seed germination over the five weeks, compared to both negative (0% w/w) and positive (s-metolachlor) CTLs (**Fig. 1B**), reaching, at the end of the experiment, an inhibition of seed germination of 63%, compared to the negative CTL (**Fig. 1B**).

Although the number of germinated seeds remained high throughout the experiment with the s-metolachlor treatment (**Fig. 1A** and **B**), the resulting seedlings quickly started to die over time, leading to a 96% decrease in the percentage of viable plants in the last week of treatment (**Fig. 2**).

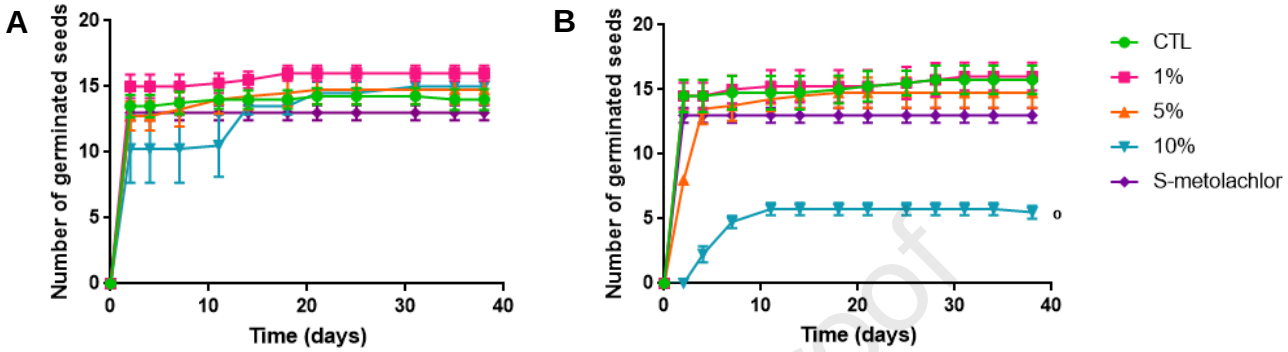


Fig. 1 – Number of germinated seeds over the five weeks of treatments with deionised water (CTL), s-metolachlor, and different concentrations [1, 5, and 10% (w/w)] of fresh leaves (A) and dried leaves (B) incorporated into the soil. Results are expressed as mean ± SD. º refers to statistically significant differences ($p \leq 0.05$) between treatments and the control (CTL), for the last week.

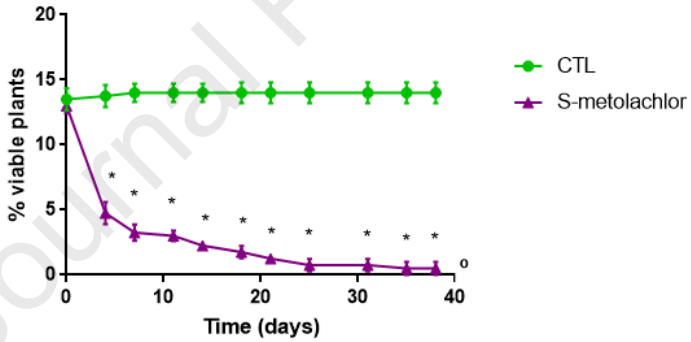


Fig. 2 – Percentage of viable plants over the five weeks of treatments with deionised water (CTL) and s-metolachlor. Results are expressed as mean ± SD. º refers to statistically significant differences ($p \leq 0.05$) between s-metolachlor and the control (CTL), for the last week; * refers to statistically significant differences ($p \leq 0.05$) between weeks, for each treatment and the CTL.

3.1.2. Macroscopic phytotoxicity symptoms

The addition of FL and DL into the soil induced leaf chlorosis and repressed plant growth and development in a dose-dependent manner, compared to the negative CTL plants (**Fig. 3**). From both leaf fragments, the inclusion of DL into the soil at 10% (w/w) had the

most accentuated inhibitory effects, preventing seed germination and the development of any leaves besides the cotyledons, which displayed a yellow-brown colour. This effect was different from that obtained with the positive CTL, in which plants suffered a great reduction in number, and those that survived became dwarf and presented anomalous leaf morphology, as shown in **Fig. 4**.

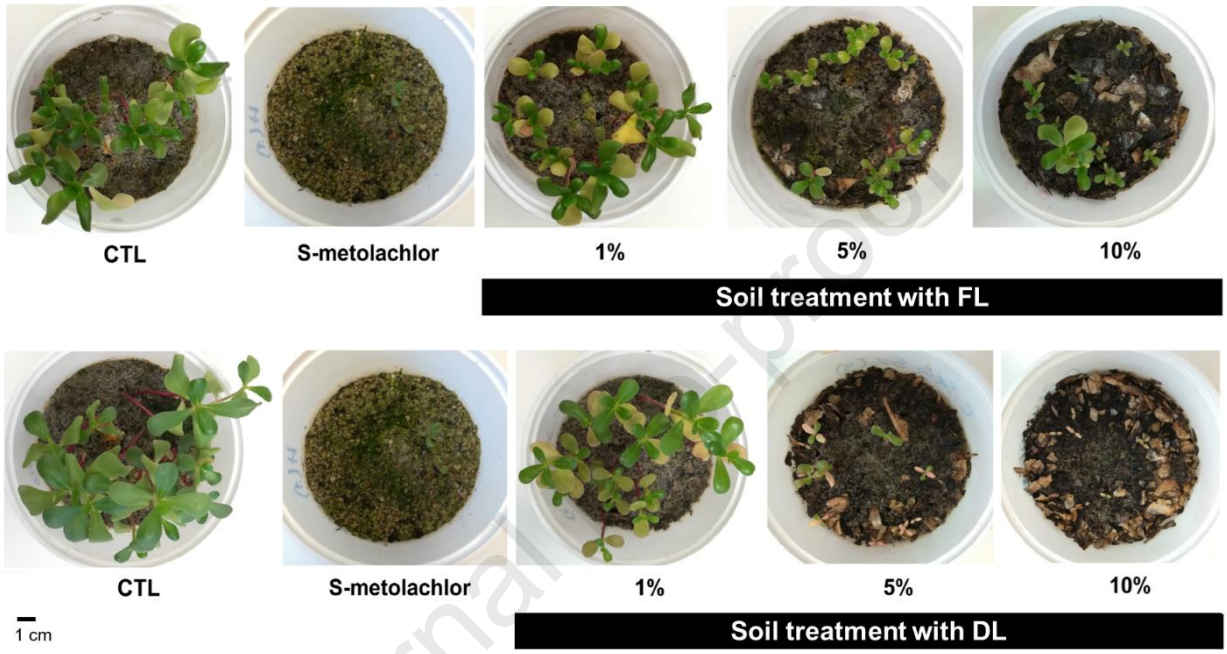


Fig. 3 – Macroscopic phytotoxicity symptoms of purslane plants after five weeks of treatments with deionised water (CTL), s-metolachlor, and different concentrations [1, 5, and 10% (w/w)] of fresh leaves (FL) and dried leaves (DL) incorporated into the soil.

Fig. 4 – Phenotype of purslane seedlings grown for five weeks in the soil treated with s-metolachlor.

3.1.3. Biometric parameters and biomass production

The soil incorporation of FL and DL impaired the growth of purslane seedlings in a concentration-dependent manner, as confirmed by the significant interaction between the factors “type of incorporated leaves” and “applied concentration” of the two-way ANOVA for all the growth-related parameters. This suggests that the pre-emergent herbicidal potential of eucalyptus leaves depends on whether the leaves added to the soil were fresh or dried as well as their concentration. Indeed, the application of FL to the soil at 1%, 5%, and 10% (w/w) decreased shoot length by about 30%, 52%, and 49%, respectively, compared to the CTL situation (**Table 1**). Regarding DL, shoot length was also affected, but in a dose-dependent manner, with reductions of approximately 17%, 68%, and 80%, in relation to the CTL (**Table 1**). About shoot biomass, a decrease of 46%, 73%, and 74% was registered with the incorporation of FL at 1%, 5%, and 10% (w/w) into the soil, respectively, compared to CTL (**Table 1**). On the other hand, the soil treatment with DL at 5% and 10% (w/w) reduced the shoot biomass of purslane plants by 94% and 96%, respectively, in comparison to CTL plants (**Table 1**).

Table 1- Shoot and root length and biomass of the purslane plants treated for five weeks with deionised water (CTL), s-metolachlor, and different concentrations [1, 5, and 10% (w/w)] of fresh leaves (FL) and dried leaves (DL) incorporated into the soil. Since the number of individuals that survived s-metolachlor treatment was considerably low ($n < 3$), it was not possible to obtain a robust statistic for this treatment. Results are expressed as mean ± SD. Different letters (capital letters: shoots; lowercase letters: roots) after the numbers denote significant differences ($p \leq 0.05$), according to Tukey’s post hoc test.

Treatment		Length (cm)		Fresh weight (g)	
		Shoot	Root	Shoot	Root
CTL		7.81 ± 0.49 A	9.02 ± 0.70 a	0.35 ± 0.02 A	0.030 ± 0.005 a
S-met		-	-	-	-
FL	1% (w/w)	5.43 ± 0.71 B	8.97 ± 1.64 a	0.19 ± 0.06 B	0.023 ± 0.005 a
	5% (w/w)	3.79 ± 0.18 C	5.36 ± 0.52 b	0.093 ± 0.05 C	0.0090 ± 0.0026 b
	10% (w/w)	3.95 ± 0.66 C	4.93 ± 1.53 b	0.089 ± 0.02 C	0.0085 ± 0.0015 b
CTL		7.34 ± 0.15 A	6.47 ± 0.39 a	0.27 ± 0.04 A	0.028 ± 0.002 a

S-met		-	-	-	-
DL	1% (w/w)	6.07 ± 0.47 B	6.07 ± 0.47 a	0.27 ± 0.029 A	0.028 ± 0.004 a
	5% (w/w)	2.36 ± 0.29 C	1.73 ± 0.76 b	0.022 ± 0.007 B	0.0047 ± 0.0015 b
	10% (w/w)	1.44 ± 0.43 C	1.49 ± 0.14 b	0.016 ± 0.008 B	0.0013 ± 0.0003 b

Concerning root length, only the two highest concentrations of both treatments (DL and FL) significantly altered this growth parameter (**Table 1)**. While the incorporation of FL at 5% and 10% (w/w) into the soil decreased root elongation by about 41% and 45%, respectively (**Table 1**), the soil treatment with DL at the same concentrations, induced a greater reduction (73% and 77%, respectively), compared to CTL plants (**Table 1**). Likewise, soil treatment with FL or DL at the two highest concentrations reduced root fresh biomass of purslane plants by approximately 70% and 71% (FL) and 84% and 96% (DL), compared to CTL plants (**Table 1**).

However, the effects of both types of eucalyptus leaves in shoot and root length and fresh biomass were not comparable to those obtained with s-metolachlor, where plant growth was completely repressed (**Table 1**).

3.1.4. Cell viability

As shown in **Fig. 5A**, the incorporation of FL into the soil at 5% and 10% (w/w) reduced the cell viability of purslane plants, showing effects quite similar to those of s-metolachlor. Likewise, soil treatment with DL at the two highest concentrations induced severe cell damage and showed a pattern of cell death close to that of s-metolachlor (**Fig. 5B**).

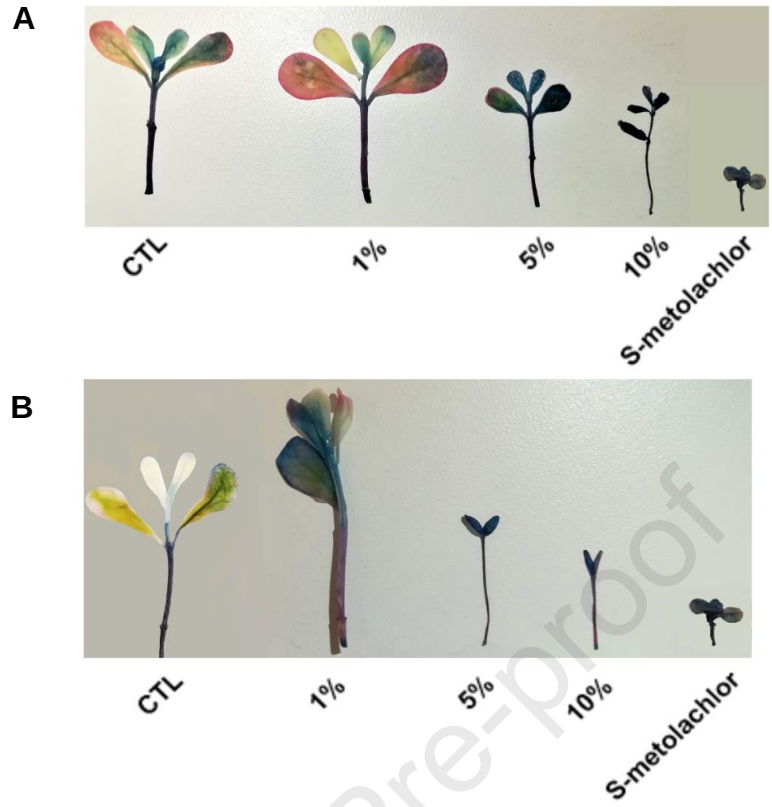


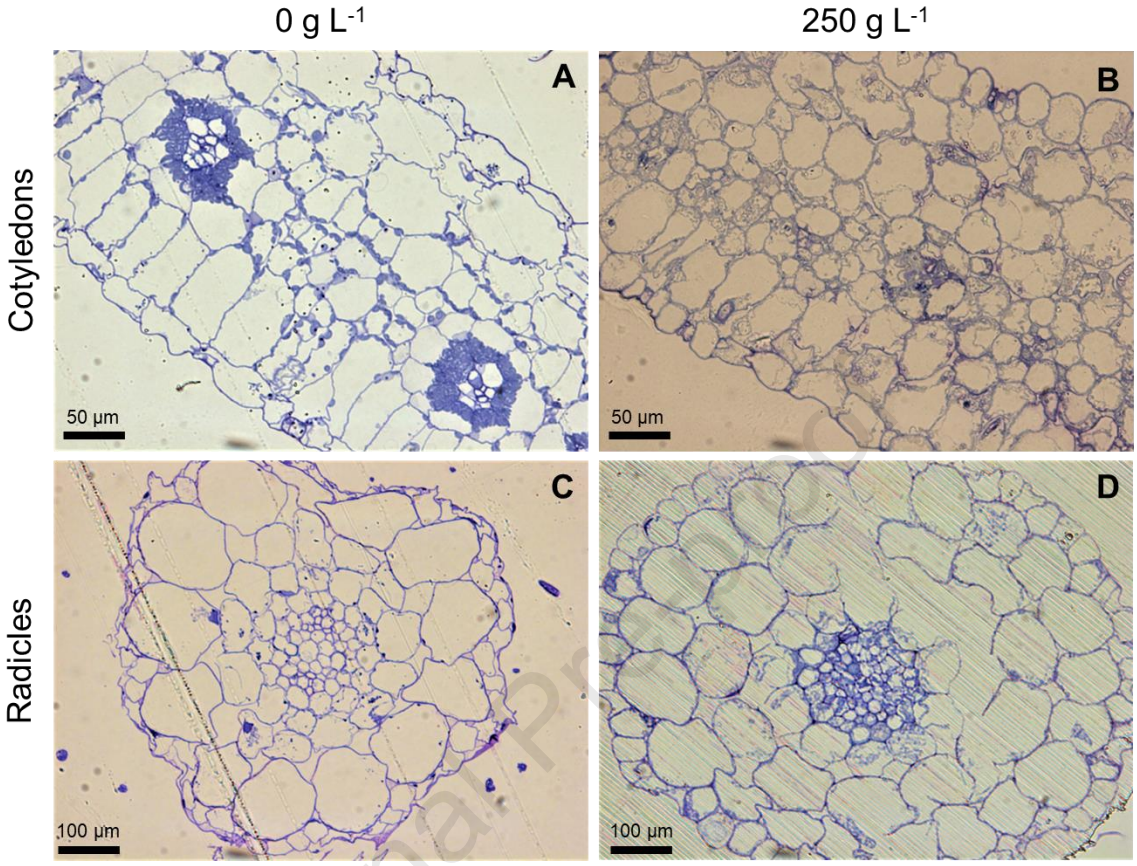
Fig. 5 – Cell death staining in shoots of purslane plants treated for five weeks with deionised water (CTL), s-metolachlor, and different concentrations [1, 5, and 10% (w/w)] of fresh (A) and dried (B) leaves incorporated into the soil.

3.2. Potential phytotoxic effects induced by the pre-emergent application of young eucalyptus leaves

3.2.1. Cell structure analysis

In general, the germination of purslane seeds in the medium containing the extract prepared from DL of eucalyptus caused a strong cellular disorganization, leading to the loss of the characteristic anatomy of C4 plants (**Fig. 6**). More specifically, in cotyledons, in comparison with those from CTL seedlings (0 g L^{-1}; **Fig. 6A**), the treatment with the eucalyptus extract led to an atypical anatomical pattern, with the loss of the Kranz anatomy (in which the mesophyll cells are arranged in a ring-like shape around bundle-sheath cells), and absence of chloroplasts in the bundle sheath cells being observed (**Fig. 6B**). Concerning radicles, the exposure to the extract induced the structural

366 disorganization of vascular tissue cells, whereas peripheral root cells preserved their
367 anatomy (**Fig. 6D**).



368 **Fig. 6 –** Structure of the cotyledons (A and B) and radicles (C and D) of purslane seedlings germinated in
369 MS medium containing deionised water (0 g L^{-1}) and an aqueous extract prepared with dried eucalyptus
370 leaves (250 g L^{-1}). (A) mesophyll cells showing the typical Kranz anatomy of C4 plants; (B) mesophyll cells
371 presenting an atypical pattern of organization, without chloroplasts aligned next to the bundle sheath cells;
372 (C) characteristic anatomy of primary radicle cells; (D) disorganized primary radicle cells.

373 **3.2.2.** Ultrastructure analysis

374 The ultrastructural effects of eucalyptus leaf extract exposure on purslane seedlings
375 were evaluated to better understand the response of mesophyll and radicle cells to the
376 extract treatment. Seedlings exposed to the eucalyptus leaf extract showed significant
377 subcellular disorganization in both analysed organs (**Figs. 7** and **8**). In cotyledons, the
378 treatment with the aqueous extract caused evident alterations in mesophyll cells, which
379 displayed an abundance of large lipid droplets (**Fig. 7d-f**), which were observed only
380 sporadically in control cells (**Fig.7a-c**). Additionally, the screening of a high number of

mesophyll cells of treated seedlings also showed the presence of cellular debris next to cell walls, a lower number of mitochondria and chloroplasts with decreased size, but with huge starch grains (**Fig. 7d-f**), features that were not observed in control cells (**Fig. 7a-c**). As illustrated in **Fig. 7c**, in cotyledons of control seedlings the presence of proteinoplasts was noted, but they were absent in cells of eucalyptus extract-treated seedlings (**Fig. 7d-f**).

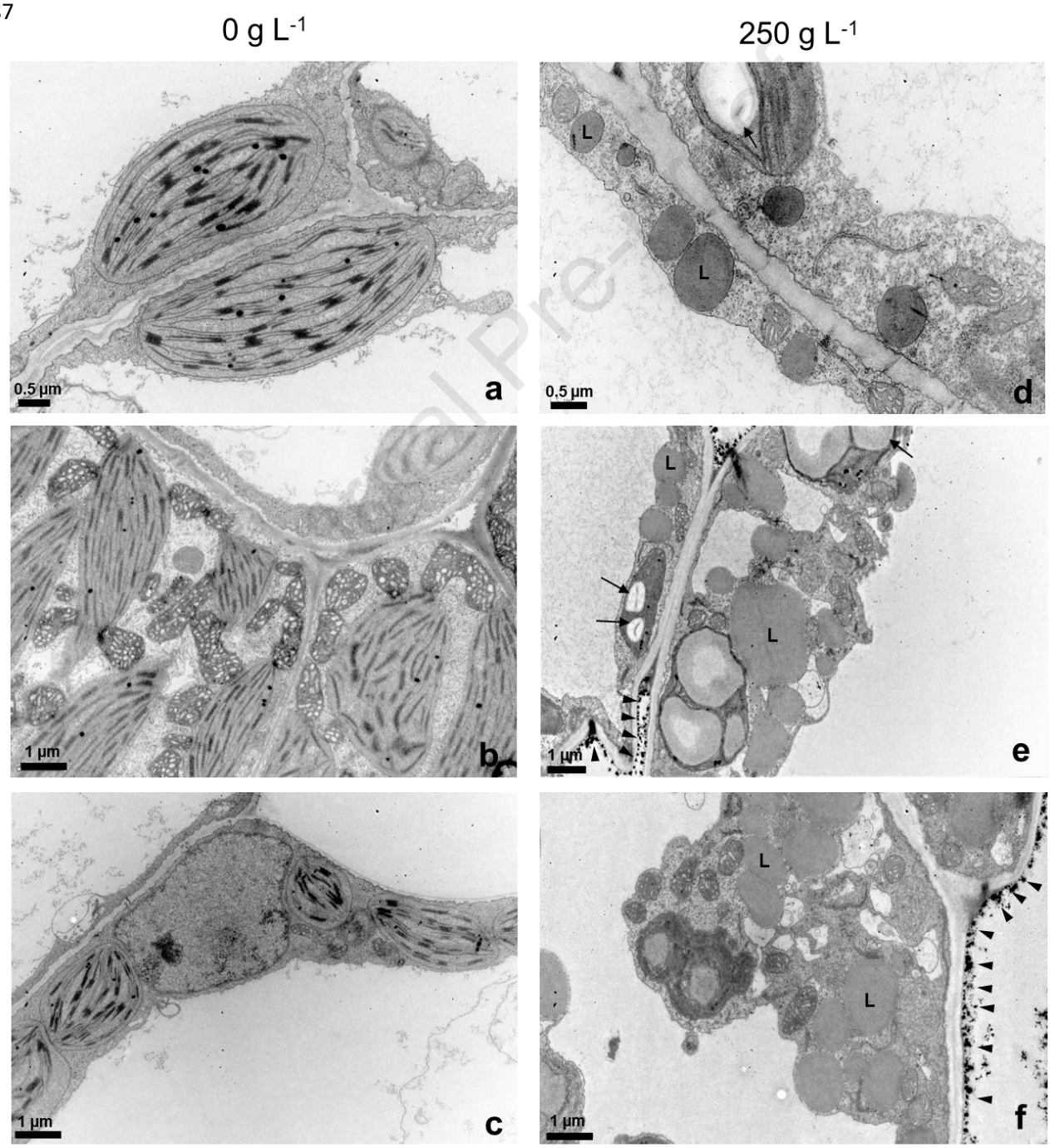
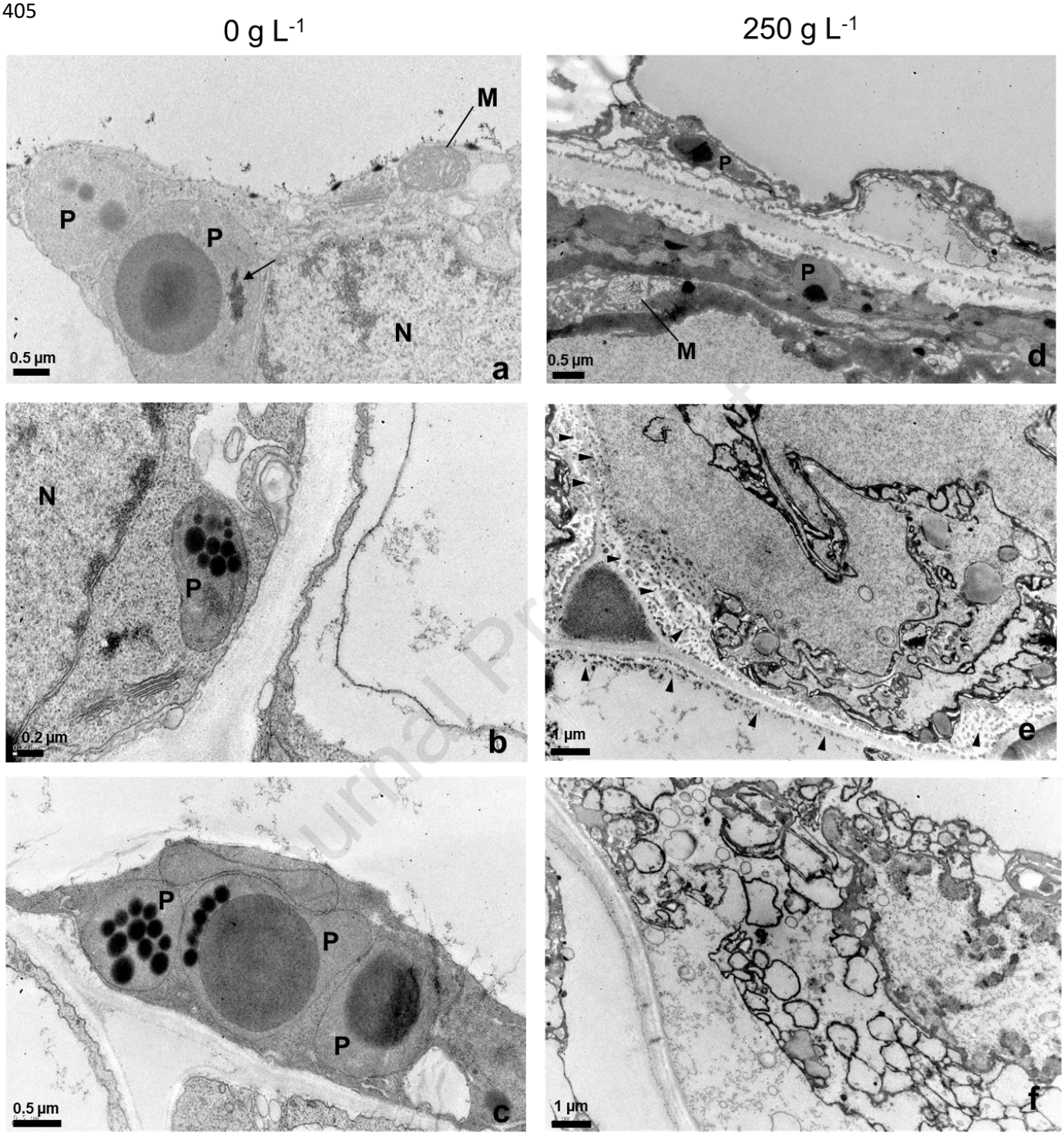


Fig. 7 – Cell ultrastructure of mesophyll cells of cotyledons of purslane seedlings germinated in MS medium containing deionised water (0 g L^{-1}) and an aqueous extract prepared with dried eucalyptus leaves (250 g L^{-1}). (a, b, c) Region of control mesophyll cells displaying well-preserved chloroplasts, mitochondria, and nucleus. (d, e, f) Portions of treated mesophyll cells, showing several lipid droplets (L), a feature only observed in treated mesophyll cells, which also displayed chloroplasts with large starch grain deposition (arrows; 7d, e) and accumulation of cellular debris (arrowheads; 7e, f) at the periphery of cells near the cell wall (arrowheads; 7f) or even in the intercellular space (arrowheads; 7e). In treated cells, loss of cell integrity and organization was commonly observed (7e, f).

The exposure of seedlings to the eucalyptus extract resulted in the loss of structural organization of radicle cells, clearly indicating that their integrity was greatly affected by the extract (**Fig. 8d-f**). Upon observation of numerous ultra-thin sections of cells treated with the extract, severe damage to cellular components was perceived, as well as the deposition of lipid droplets and aggregates of cellular debris resulting from organelle lysis (**Fig. 8d-f**). These features were not observed in control cells, which exhibited cellular preservation (**Fig. 8a-c**). Like mesophyll cells in the control group, proteinoplasts were present in radicle cells (**Fig. 8a-**c). However, in cells exposed to eucalyptus extract, the occurrence of proteinoplasts was only occasional (**Fig. 8d-f**).

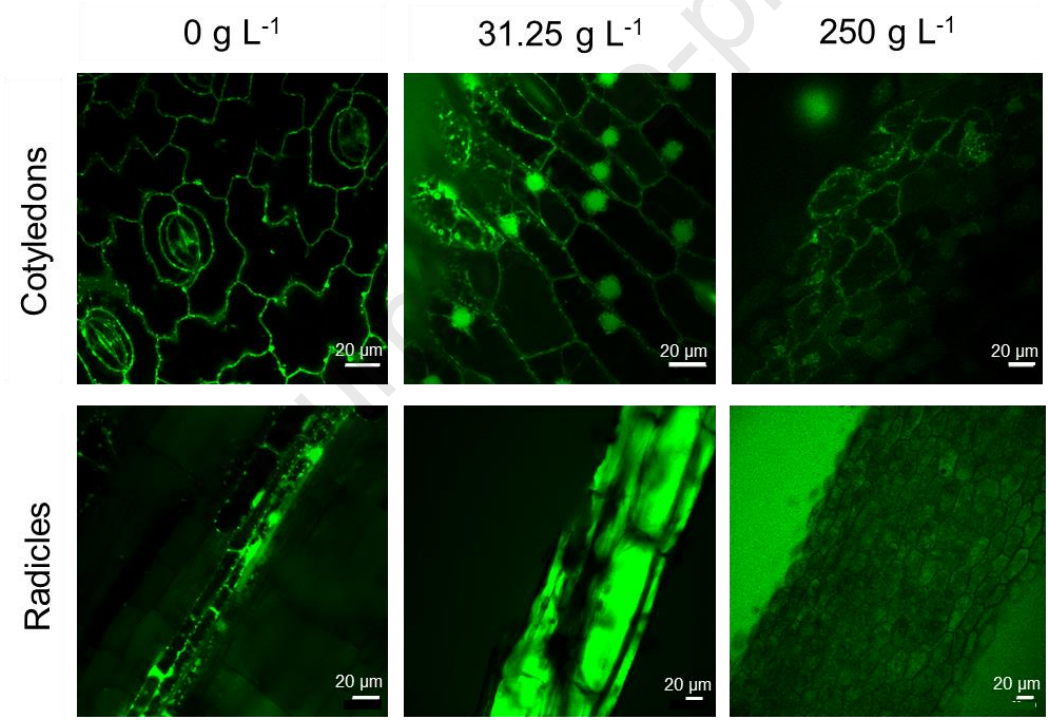
405



406 **Fig. 8** – Cell ultrastructure of radicle cells of purslane seedlings germinated in MS medium containing
407 deionised water (0 g L^{-1}) and an aqueous extract prepared with dried eucalyptus leaves (250 g L^{-1}). (a, b, c)
408 Portions of control cells with proteinoplasts (P), one of them containing ferritin (arrow; 8a), nucleus (N; 8a,
409 b), and well-preserved mitochondria (M; 8a); (d, e, f) Regions of treated radicle cells showing a high degree
410 of disorganization and loss of cell integrity. Negative effects of the treatment on mitochondria (M; 8d), on the
411 accumulation of cellular debris near the cell wall (arrowheads; 8d, e) and lipid droplets are noticeable.

412 **3.2.3.** ROS *in vivo* detection

413 The exposure of purslane seedlings to the lowest concentration of the eucalyptus extract
414 (31.25 g L^{-1}) induced an increase in H_2O_2 staining signal intensity in both cotyledons and
415 radicles, while exposure to the highest concentration (250 g L^{-1}) of the extract resulted
416 in a decrease in signal intensity in both organs, compared to the control situation (**Fig.**
417 **9**). In contrast, exposure to 31.25 g L^{-1} of the eucalyptus extract led to a decrease in $O_2^{\cdot-}$
418 staining signal intensity in both cotyledons and radicles. However, in seedlings
419 germinated in the medium containing the highest concentration of eucalyptus extract, the
420 signal intensity of $O_2^{\cdot-}$ staining increased in both organs compared to the controls (**Fig.**
421 **10**).



431 **Fig. 9** – H_2O_2-associated fluorescence [by using 2',7'-dichlorofluorescein diacetate (DCF-DA)] of cotyledons
432 (upper images) and radicles (bottom images) of purslane seedlings treated with deionised water (0 g L^{-1})
433 and two concentrations of an aqueous extract prepared with dried eucalyptus leaves (31.25 and 250 g L^{-1}).
434 Note the increased signal intensity of H_2O_2 staining in both cotyledons and radicles when exposed to the
435 31.25 g L^{-1} concentration of the eucalyptus extract, while the exposure to the highest concentration (250 g
436 L^{-1}) resulted in decreased signal intensity in both organs.

437

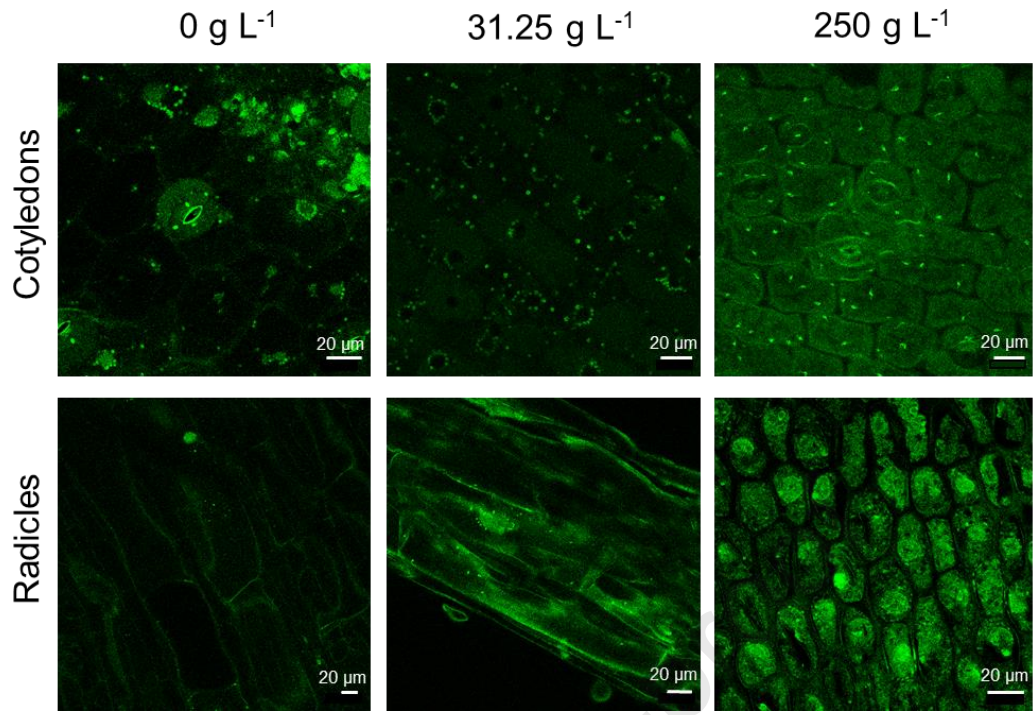


Fig. 10 – $O_2^{\cdot-}$-associated fluorescence [by using dihydroethidium (DHE)] of cotyledons (upper images) and radicles (bottom images) of purslane seedlings treated with deionised water (0 g L^{-1}) and two concentrations of an aqueous extract prepared with dried eucalyptus leaves (31.25 and 250 g L^{-1}). It is noticeable a decrease and an increase in the signal intensity of $O_2^{\cdot-}$ staining in both cotyledons and radicles upon the exposure to the lowest and the highest concentration of the eucalyptus extract, respectively.

4. DISCUSSION

4.1. Young eucalyptus leaves, applied as a soil amendment, have great pre-emergent biocidal potential, especially when prepared with DL

The soil incorporation of plant debris from species with allelopathic properties, resembling naturally occurring litter decomposition, can represent a sustainable strategy to prevent weed proliferation (El-Rokiek et al., 2011). In forest ecosystems, this leaf layer gradually releases the allelochemicals in the soil rhizosphere, inhibiting seed germination, and the subsequent development and growth of other plants . Although this natural property of eucalyptus leaves seems promising, especially in terms of economic costs, only a couple of authors have investigated this strategy using *E. globulus* biomass (El-Rokiek et al., 2011; Puig et al., 2013). While El-Rokiek et al. (2011) used DL, Puig et al. (2013) incorporated FL into the soil. Both studies concluded that the incorporation of

459 mature *E. globulus* leaves into the soil can be used for weed control. However, there is
460 a lack of knowledge regarding the pre-emergent biocidal potential of leaves from young
461 *E. globulus* trees and their potential cellular targets. In order to address this gap, fresh
462 (FL) and dried (DL) eucalyptus leaves were incorporated into the soil at different
463 percentages [1, 5, and 10% (w/w)] along with purslane seeds to evaluate the herbicidal
464 potential of young eucalyptus trees' biomass in a pre-emergent scenario.

465 The incorporation of FL into the soil did not detain an efficient pre-emergent
466 biocidal activity against *P. oleracea.* The number of germinated seeds with the FL
467 treatment was very similar to that of the negative CTL, indicating that the presence of
468 the leaves in the soil did not inhibit seed germination. Still, the presence of FL in the soil
469 severely affected the growth and development of purslane seedlings in a dose-
470 dependent manner – the higher the concentration of leaf fragments, the lower the growth
471 performance of seedlings. In fact, shoot elongation and biomass production were found
472 to greatly decrease upon exposure to increasing concentrations of the FL. However, the
473 same trend was not observed for root-related growth parameters, which exhibited similar
474 decreases when FL were applied at both 5% (w/w) and 10% (w/w). This reduction in root
475 growth was further supported by a decrease in cell viability, particularly at the higher
476 concentrations of 5% and 10% (w/w). These findings agree with the study of Abdelmigid
477 and Morsi (2017), where it was shown that allelochemicals released from fresh mature
478 *E. globulus* leaves caused DNA breakages, leading to senescence and programmed cell
479 death (PCD) in soybean seedlings, when fragmented leaves were incorporated into the
480 soil at different concentrations. Moreover, in response to allelopathic stress, various
481 types of proteases involved in PCD were also upregulated (Abdelmigid and Morsi, 2017).

482 Concerning the treatment with DL, a strong biocidal potential in a pre-emergent
483 context was verified, since seed germination was considerably inhibited when DL were
484 incorporated into the soil at 10% (w/w). In addition to the inhibition effect of seed
485 germination, the inclusion of DL into the soil at 10% (w/w) repressed the growth and
486 development of purslane seedlings in such a way that they did not develop any true

leaves, besides cotyledons. Furthermore, regardless of the applied concentration, the number of germinated seeds in eucalyptus leaf-treated soils was also comparable to those treated with s-metolachlor at the recommended application dose. S-metolachlor is a selective herbicide that belongs to the chemical family of chloroacetanilides (Maronić et al., 2018). This synthetic herbicide can be applied in both pre-emergent and early post-emergent stages of weeds (Liu et al., 2012b). In fact, s-metolachlor characteristically interferes in the earliest metabolic processes of seedling growth (Sherwani et al., 2015), thus explaining the constant number of germinated seeds and the pronounced decrease in the percentage of viable plants throughout the experiment. On the other hand, the synthetic herbicide not only significantly impaired the growth of *P. oleracea* seedlings, but also induced caused abnormal morphology. These divergent outcomes may rely on distinct modes-of-action between the eucalyptus-based herbicide and the chemical herbicide. S-metolachlor, classified as a shoot-growth inhibitor, exerts its effects on plant cells by binding to acetyl co-enzyme A and molecules containing sulfhydryl groups. This binding suppresses the production of very long-chain fatty acids (VLCFA) during seedling growth (Sherwani et al., 2015).

Therefore, s-metolachlor disrupts cell development by inhibiting cell division and elongation (Bach and Faure, 2010). In turn, the phenolic compounds present in plant-based herbicides, when applied in a pre-emergence context, can reduce the activity of α-amylase in seeds, thereby impairing nutrient supply for the embryo and affecting seed germination (Radhakrishnan et al., 2018). This could potentially explain the significant inhibition of seed germination observed when DL were incorporated into the soil at the concentration of 10% (w/w), in comparison with the effect produced by the incorporation of FL. While the chemical composition of an extract obtained from FL from young *E. globulus* trees revealed a great content of compounds, like carbohydrates, ellagitannins, hydroxycinnamic acids, and terpenoid derivatives, the drying process of eucalyptus leaves enriched the aqueous extract prepared with dried leaves of young *E. globulus*

514 with low molecular weight compounds with known allelopathic activity like gallic and
515 benzoic acids, fatty acid derivatives, and flavonoids (Pinto et al., 2022).

516 Furthermore, due to the thin cell layers of primary root surfaces, the allelochemicals
517 present in bioherbicides can easily enter into root cells, affecting the cell cycle and cell
518 membranes' ultrastructure, as discussed below, which results in the inhibition of root
519 growth (Radhakrishnan et al., 2018). In fact, root length and biomass were severely
520 impaired upon the incorporation of DL into the soil at the highest concentrations. In this
521 way, the inhibition of shoot growth in plants grown in DL-treated soils [10% (w/w)] may
522 have occurred as a consequence of higher absorption of phytotoxic compounds by roots,
523 and/or impairment of nutrient uptake caused by the presence of allelochemicals in the
524 soil (Radhakrishnan et al., 2018), similar to what was observed with a post-emergent
525 treatment of purslane seedlings with an aqueous extract prepared with DL (Pinto et al.,
526 2021). Therefore, the treatment with DL at 10% (w/w) exhibited stronger herbicidal
527 activity in a pre-emergent context compared to the soil application of FL at the same
528 concentration. However, this activity differed from that of the synthetic herbicide, as they
529 affected different stages of weed development: s-metolachlor primary hindered early
530 seedling growth, while the eucalyptus-based herbicide primary impeded seedling
531 development.

532 4.2. Allelochemicals released from young eucalyptus leaves profoundly altered cellular
533 and subcellular structure and induced severe redox disorders

534 To unravel the potential mode-of-action and cellular targets of this pre-emergent
535 bioherbicide on the initial developmental stages of purslane seedlings, a complementary
536 experiment was conducted where purslane seeds germinated in a nutritive medium
537 containing an aqueous extract prepared with DL. The metabolomic profile of this extract
538 had been extensively characterized in a previous study, which revealed its richness in
539 phenolic acids, like gallic acid and benzoic acid, flavonoids, fatty acids, organic acids,

and amino acids (Pinto et al., 2022). To analyse the cellular and subcellular targets of these allelochemicals present in eucalyptus leaves, cotyledons and seedling radicles were studied at histological and ultrastructural levels. Furthermore, the production of two important ROS, H_2O_2 and $O_2^{-\cdot}$, on both organs exposed to the extract was detected *in vivo*, to assess if the eucalyptus leaf extract induces a burst in ROS accumulation that could explain the overall herbicidal activity.

Synthetic and plant-based herbicides usually interfere with weed growth by inducing severe oxidative bursts of ROS that the plant antioxidant system cannot surpass as a primary target or as a consequence of their mode-of-action (Kaur, 2019;Šoln et al., 2022). This condition can lead to serious oxidative stress disorders, like the degradation of membrane lipids, thus causing lipid peroxidation and strong cell membrane damage, which, in turn, can promote programmed cell death (Šoln et al., 2022). The contrasting patterns of H_2O_2 and $O_2^{-\cdot}$ accumulation in both organs of purslane seedlings treated with low and high concentrations of the eucalyptus extract indicate that at low doses, phytochemicals may induce ROS production at levels that the antioxidant system can effectively counteract. On the other hand, the exposure to high concentrations of the eucalyptus extract may lead to an overwhelming burst of ROS, surpassing the capacity of the antioxidant system. This explains the reduced intensity of the H_2O_2-associated signal and the great accumulation of $O_2^{-\cdot}$ in both organs of purslane seedlings treated with the eucalyptus extract. These results were supported by the study of Babula et al. (2009), in which cultured tobacco cells treated with naphthoquinones, such as juglone and plumbagin, exhibited a clear overproduction of $O_2^{-\cdot}$. Additionally, it has been reported that phenolic acids like benzoic acid and cinnamic acid, major components of the eucalyptus extract used in the present study (Pinto et al., 2022), considerably impaired the growth of *Arabidopsis thaliana* and *Cucumis sativus* plants, by inducing great redox disorders, characterized by ROS overproduction (Ding et al., 2007;Zhang et al., 2018).

In turn, this severe ROS oxidative burst induced by the allelochemicals present in the eucalyptus extract can explain the marked loss of cell integrity and organization registered in both organs of purslane seedlings treated with the extract. In fact, the exposure of *P. oleracea* to the eucalyptus extract led to the loss of the typical leaf anatomy of C4 plants, known as Kranz anatomy (Ferrari et al., 2020), thus showing that the photosynthetic process could have been majorly compromised. In fact, the number of chloroplasts of treated seedlings was strongly reduced and their morphology visibly altered, presenting decreased size and huge starch grains. Accordingly, flavonoids, one of the major phytochemical classes present in the eucalyptus extract, have been reported to affect the photosynthetic activity of plants and to disrupt adenosine triphosphate (ATP) production, leading plants to create energy supplies in the attempt of guaranteeing their survival (Palma-Tenango et al., 2017).

Besides impairing photosynthetic activity, compounds belonging to chemical classes of phenolic acids and flavonoids can affect the respiration process by reacting with mitochondrial membranes, leading to the death of mitochondria (Palma-Tenango et al., 2017;Radhakrishnan et al., 2018;Anwar et al., 2021). As a matter of fact, cotyledon cells of treated seedlings showed decreased numbers of mitochondria. However, the few remain had a similar morphology to the ones present in control cells. Besides preserved membranes, mitochondrial cells of both situations presented dilated cristae, indicative of a high respiration activity, which is a very common feature in seedlings of such young age.

Radicles of *P. oleracea* exposed to the eucalyptus extract exhibited significant cellular and subcellular alterations, characterised by pronounced cell disorganization and disruption of cellular content, particularly in central cells. These central cells undergo differentiation to form conducting vessels, which possibly make them more vulnerable to the phytochemicals present in the eucalyptus extract, when compared to cortical cells. Therefore, the observed ultrastructural damage can be attributed to the direct effect of

allelochemicals present in eucalyptus extract, which interfere with root nutrient uptake (Palma-Tenango et al., 2017;Radhakrishnan et al., 2018a;Šoln et al., 2022). Additionally, the substantial burst of ROS induced by eucalyptus metabolites in purslane radicles may contribute to the observed damage.

The highly reactivity of ROS can target lipids of cell membranes and organelles (Anwar et al., 2021;Šoln et al., 2022), contributing to the observed oxidative damage. In fact, the accumulation of cellular debris in the cytoplasm of radicle cells exposed to the eucalyptus extract might result from the induced organelle lysis. In line with this, Cruz-Ortega et al. (1998) demonstrated that the allelochemical stress caused by the application of an aqueous extract of *Sicyos deppei* resulted in significant cell disorganization and reduced cell differentiation in the roots of *Phaseolus vulgaris.*

Furthermore, the exposure of purslane seedlings to the eucalyptus extract led to the accumulation of lipid droplets in both organs and a reduction in the number of proteinoplasts in radicles, with their absence in cotyledons. Lipid droplets, produced in the endoplasmic reticulum, play a crucial role in transporting neutral lipids for energy metabolism and membrane synthesis (Huang et al., 2019). They are involved in cell signalling and respond to various stresses, including nitrogen depletion, by participating in lipid membrane restoration (Huang et al., 2019).

The significant accumulation of lipid droplets in purslane seedlings treated with the eucalyptus aqueous extract may represent a defence mechanism of plant cells against the toxicity caused by the release of allelochemicals from eucalyptus DL. In fact, the accumulation of lipid droplets has been reported as a common response of cells to the application of plant-based herbicides (Radhakrishnan et al. (2018). Proteinoplasts are organelles commonly found in *P. oleracea* cells, reflecting the species' richness in proteins and fatty acids like omega-3 (Petropoulos et al., 2019). The oxidative stress induced by the eucalyptus extract exposure may have resulted in the degradation of

proteins and lipids, which are primary targets of ROS attack under allelochemical-induced stress conditions (Anwar et al., 2021;Šoln et al., 2022), potentially leading to proteinoplast lysis in both organs. Indeed, at the ultrastructural level, a notable difference observed in root cells of treated seedlings compared to control cells was a reduction in the number of proteinoplasts and an increased presence of cellular aggregate deposits. These deposits likely formed as a result of organelle lysis, indicating the occurrence of early senescence events in these cells.

5. CONCLUSIONS

In conclusion, our findings demonstrated, for the first time, the promising herbicidal potential of young *E. globulus* leaves as a pre-emergent treatment against *P. oleracea*. Particularly, the application of dried leaves at the concentration of 10% (w/w) exhibited great herbicidal activities. Additionally, our results emphasize that this eucalyptus-based herbicide exerts its effects on purslane physiology by inducing excessive production of ROS, leading to severe cell disorganization and loss of cell integrity in both cotyledons and radicles.

To further enhance our understanding of the biocidal activity of this eucalyptus-based herbicide, it is crucial to conduct tests under real environmental conditions and evaluate its potential non-target effects on soil organisms and crops. These studies will provide valuable insights into the efficacy and safety of this herbicide for practical applications.

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HIGHLIGHTS:

1. Dried leaves of young *E. globulus* had the greatest pre-emergent herbicidal activity;
2. Allelochemicals caused redox disorders in purslane cotyledons and radicles;
3. Phytochemicals induced the loss of cell organization and integrity;
4. Dried leaf soil incorporation can represent an alternative for weed control.

Author's contributions

MP: conceptualization, experimental design, investigation, writing of original draft, and manuscript review and editing. BS and MM: investigation. CP: investigation, manuscript review and editing, supervision, and resources. CS: conceptualization, experimental design, investigation, manuscript review and editing, and supervision. FF: conceptualization, experimental design, investigation, manuscript review and editing, supervision, resources, project administration, and funding acquisition.

Declaration of interests

☐ 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.

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