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BIOTECHNOLOGY

Phytotoxic and mito-depressive effects of *Rosmarinus officinalis* essential oil

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ABSTRACT. Allelopathy is a phenomenon involving beneficial or adverse effects from one plant to another by releasing compounds called allelochemicals. Essential oils, which are volatile substances responsible for plant aromas, may play a role in this phenomenon. This study aimed to evaluate the phytotoxic and cytotoxic effects of rosemary (*Rosmarinus officinalis*) essential oil. Phytotoxic effects of the oil (1 µL, 5 µL, 10 µL, and 20 µL) were assessed on germination and initial growth of two target species: a crop (*Lactuca sativa* L., lettuce) and a weed (*Eragrostis plana*, capim-annoni-2). The evaluated variables were germination rate, speed of germination, root length, and shoot length. The cytotoxic effects of essential oil (0.5 µL, 1 µL, and 10 µL) were assessed on *L. sativa* root tips, evaluating its impact on mitotic and metaphasic indexes as well as the frequency of each mitotic phase. The essential oil was extremely harmful to *L. sativa* germination and initial growth. The volatiles also caused an inhibitory, dose-dependent effect in *E. plana* germination and strongly inhibited both root and shoot growth of the species at the highest amounts tested. Rosemary essential oil interfered with the cell division of *L. sativa*, resulting in decreased mitotic and metaphasic indexes. Analysis of the frequency of each mitosis phase indicates that volatiles inhibited root growth by delaying cell division. These findings suggest that rosemary essential oil could serve as a potential bioherbicide.

Keywords: Allelopathy; cytogenetics; germination; growth; volatiles.

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Introduction

Allelopathy is a natural interference mechanism whereby a plant produces compounds, known as allelochemicals, which can benefit or harm other organisms when released into the environment (Rice, 1984). Inhibitory effects on germination and plant growth are typically associated with allelopathy, indicating the allelopathic potential of the tested compounds (Silva et al., 2017). These processes are crucial for understanding plant interactions in both natural environments and agroecosystems. In agricultural systems, allelopathic interference can be analyzed regarding the desirable beneficial effects on crops or adverse effects on weeds. Natural products produced by plants have been tested to provide new opportunities for diversifying control as eco-friendly herbicides (bioherbicides) in agriculture, thereby reducing environmental contamination (Verdeguer et al., 2020; Werrie et al., 2020).

Visible morphological changes in response to allelochemicals could be due to effects on the cellular or molecular level (Dragoeva et al., 2015). Cell division can be comprehended as a necessity for cell growth in organisms, and this uniform division of the cell components allows for their balanced growth (Sánchez-Moreiras et al., 2006). Tissue growth in plants depends on a normal mitotic process. The mitotic index has been used as a parameter to assess the cytotoxicity of several agents including allelochemicals (Graña, 2018). Thus, cytogenetics can be a useful tool to understand the mode of action of allelochemicals, which, in turn, is pivotal for bioherbicide development studies. However, despite this, the majority of allelopathy studies have only assessed the effects of phytotoxins on germination and plant growth, with few investigating their impact on the cellular level or elucidating their mode of action (Silva et al., 2017).

Rosmarinus officinalis L., commonly known as rosemary, is a shrub of the Lamiaceae family, originally from the Mediterranean region and cultivated in various parts of the world (González-Minero et al., 2020; Oliveira, & Veiga, 2019). Rosemary is very appreciated as a flavoring agent in foods and has been widely used in traditional medicine. Also, due to its aromatic value, *R. officinalis* secondary metabolites have been explored in cosmetics formulations (González-Minero et al., 2020) and in aromatherapy (Mank-halati et al., 2024). Rosemary essential oil has been recognized as safe for human health and the environment and its use has been approved by the USDA's Organic Program.

Essential oils are volatile substances produced by plants responsible for their aromas. Plants may produce volatile compounds in flowers, fruit peels, leaves, roots, bark, and seeds, which serve multiple ecological functions, including self-defense (Pierik et al., 2014), attraction of pollinators (Pierik et al., 2014), and acting as allelochemicals (Verdeguer et al., 2020). Essential oils extracted from rosemary are complex mixtures of volatile compounds, with predominant components being the monoterpene hydrocarbons α-pinene and camphene and the oxygenated monoterpenes 1,8-cineole, camphor, borneol, and verbenone (Napoli et al., 2015). Chemical composition of essential oils can vary according to several factors, such as climate, soil conditions, and harvesting time (Raveau et al., 2020). This may result in chemotypes, which can be defined as the same species/subspecies/variety of an organism containing different secondary metabolites or the same secondary metabolites with different quantities (Polatoglu, 2013). The main rosemary chemotypes are α-pinene, 1,8-cineole, camphor, and verbenone (González-Minero et al., 2020) Understanding the chemotype of the donor species is important because the level of phytotoxicity varies among different rosemary chemotypes (El Mahdi et al., 2020).

This work aimed to evaluate the interference of rosemary essential oil (1,8-cineole chemotype) on germination and initial growth of a crop and a weed species. The crop species is *Lactuca sativa* L. (lettuce), an edible vegetable widely used as a model in allelopathy studies due to its fast germination and its rapid and uniform initial growth (Reigosa et al., 2013). The weed is *Eragrostis plana* Ness (capim-annoni-2), an invasive plant in the grasslands of South Brazil, Uruguay, and Argentina. *Eragrostis plana* suppresses native vegetation and decreases forage quality in pastures, leading to environmental and socio-economic impacts (Guido et al., 2024), while also invading crop plantations, such as rice and cocoa. In addition, the cytotoxic effects of rosemary volatiles were evaluated on the root tips of *L. sativa*.

Material and methods

Plant material

Lactuca sativa 'Grand-Rapids' (lettuce) diaspores were purchased from a local trade. *Eragrostis plana* (capim-annoni-2) diaspores were collected at the Agronomic Experimental Station – *Universidade Federal do Rio Grande do Sul* (UFRGS) in Eldorado do Sul, Brazil, and kindly provided by Dr. Geraldo Luiz Gonçalves Soares, UFRGS, Brazil. The essential oil of *Rosmarinus officinalis* (rosemary) 1,8-cineole chemotype, obtained from a commercial source, was extracted by steam distillation of leaves.

Germination experiments

For the germination experiment, 30 diaspores were distributed in Petri dishes on filter paper containing 3 mL of distilled water. The essential oil (1 μL, 5 μL, 10 μL, and 20 μL) was applied on cotton fixed with double-sided tape at the top of the Petri dish, which was sealed with plastic film to prevent the loss of volatiles. The control group had the same conditions, but without adding the essential oil. The experiment had three repetitions per treatment. After the protrusion of the primary root (24 hours for *L. sativa* and 72 hours for *E. plana*), the number of germinated diaspores was counted every 24 hours for four and five days, respectively, for *L. sativa* and *E. plana*. At the end of the experiment, the germination rate (the percentage of germinated seeds) and the speed of germination were calculated. Speed of germination (SG) was calculated using the following formula: $SG = [N1/1+N2/2+\ldots+Nn / n]$, where N1, N2, and Nn are the cumulative numbers of seeds that germinate by times 1, 2, ..., n.

Seedling growth experiments

For the growth experiment, 20 diaspores of each target species were used following the methodology previously described for the germination assay. After germination, when the primary root had emerged, seedlings were exposed to the rosemary essential oil in the same quantities previously mentioned for the germination experiment, in three repetitions per treatment. This methodology is important to evaluate the effect of the volatiles solely on the growth process, to avoid the overlap of germination effects with growth effects. After the exposure time (three days for *L. sativa* and four days for *E. plana*), the shoot length and root length of 10 seedlings per Petri dish were measured.

Cytogenetics assays

Lactuca sativa diaspores were germinated in Petri dishes under the same conditions used for the germination assays. The essential oil was applied after the emergence of the primary root in the quantities of 0 μL (control), 0.5 μL, 1 μL, and 10 μL, as previously described. After 48 h of exposure to the volatiles, root tips were cut and fixed in a freshly prepared mixture of absolute ethanol and acetic acid (3:1, v v^{-1}). The fixed root tips were stained using the Feulgen reaction. Three replicates were performed for each treatment and scoring was performed for four roots per replicate, totaling 6,000 analyzed cells per treatment. Mitotic and metaphasic indexes were calculated as the ratio between dividing cells or metaphasic cells and the total of examined cells. The frequency of each mitotic phase was calculated as the percentage in relation to the number of cells in mitosis in the treatment.

Statistical analyses

Differences between the measured variables (germination rate, root length, shoot length, mitotic index, metaphasic index, and frequency of each mitotic phase) were compared between groups by analysis of variance (ANOVA) or Kruskal-Wallis, for each species. ANOVA was used in cases where data followed normality and/or homogeneity of variances (tested by Shapiro-Wilk and Lavene's test, respectively). When differences were significant, ANOVA was followed by Tukey's test. In cases where data did not follow the assumptions of parametric analyses, data were analyzed by Kruskal-Wallis followed by Wilcoxon signed rank test. For all analyses, a significance level of $p \leq 0.05$ was considered.

Results

Effects of rosemary essential oil on germination

Rosemary essential oil caused phytotoxic effects on both target species. Volatiles were extremely harmful to *L. sativa* germination, by inhibiting 100% of the germination process in all quantities tested (Table 1). The essential oil also caused an inhibitory, dose-dependent effect in *E. plana* germination (Table 1). Compared to the control group, 5 μL of the essential oil reduced the germination rate and speed of germination of *E. plana* by about 70%. At 10 μL and 20 μL, rosemary essential oil was even more harmful to *E. plana*, with no germination observed at 10 μL.

Table 1. Germination rate (G) and speed of germination (SG) of *Lactuca sativa* (lettuce) and *Eragrostis plana* (capim-annoni-2) exposed to different quantities of *Rosmarinus officinalis* (rosemary) essential oil.

Treatment	*Lactuca sativa*		*Eragrostis plana*	
	G (%) Mean ± SD	SG Mean ± SD	G (%) Mean ± SD	SG Mean ± SD
Control	88.78 ± 1.92 a	24.53 ± 2.13 a	96.67 ± 5.77 a	8.67 ± 0.51 a
1μL	0.00 ± 0.00 b	0.00 ± 0.00 b	81.11 ± 15.75 ab	6.72 ± 1.88 ab
5 μL	0.00 ± 0.00 b	0.00 ± 0.00 b	31.11 ± 48.11 b	2.69 ± 4.16 bc
10 μL	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 c
20 μL	0.00 ± 0.00 b	0.00 ± 0.00 b	3.33 ± 3.33 bc	0.28 ± 0.25 bc

Values with the same letter within a column do not differ significantly according to Kruskal-Wallis followed by Wilcoxon test at $p \leq 0.05$. Data are expressed as mean ± standard deviation (SD).

Effects of the essential oil on seedling growth

Rosemary volatiles negatively interfered with the initial growth of the target species (Table 2). Once again, rosemary essential oil was extremely inhibitory on *L. sativa*: from 1μL to 20 μL the essential oil inhibited 100% of shoot growth and approximately 80% of root growth in relation to the control. The essential oil at 10 μL and 20 μL strongly inhibited root and shoot growth of *E. plana*.

Cytotoxic effects of rosemary essential oil

Rosemary essential oil exhibited cytotoxicity by negatively affecting the cell division process of *L. sativa* root tips. Compared to the control group, rosemary volatiles at 1 μL and 10 μL decreased the mitotic index by about 10% (Figure 1a). All treatments reduced the metaphasic index by half compared to the control (Figure 1a).

Regarding the frequency of each mitosis phase, the treatments with 0.5 µL and 1.0 µL of essential oil increased the percentage of cells in prophase by 30% compared to the control (Figure 1b). On one hand, the treatment with 10 µL of rosemary essential oil induced a two-fold increase in the percentage of cells in anaphase compared to the control. On the other hand, the essential oil reduced the percentage of cells in metaphase. No differences were observed in the percentage of cells in telophase, the last phase of cell division.

Table 2. Shoot length (SL) and root length (RL) of *Lactuca sativa* (lettuce) and *Eragrostis plana* (capim-annoni-2) exposed to different quantities of *Rosmarinus officinalis* (rosemary) essential oil.

Treatment	*Lactuca sativa*		*Eragrostis plana*	
	SL (cm) Mean ± SD	RL (cm) Mean ± SD	SL (cm) Mean ± SD	RL (cm) Mean ± SD
Control	0.41 ± 0.12 a	1.64 ± 0.23 A	0.76 ± 0.14 A	0.81 ± 0.55 a
1µL	0.00 ± 0.00 b	0.32 ± 0.14 B	0.82 ± 0.16 A	0.92 ± 0.17 a
5 µL	0.00 ± 0.00 b	0.39 ± 0.14 B	0.64 ± 0.14 A	0.55 ± 0.16 a
10 µL	0.00 ± 0.00 b	0.35 ± 0.07 B	0.22 ± 0.03 B	0.10 ± 0.00 b
20 µL	0.00 ± 0.00 b	0.30 ± 0.12 B	0.00 ± 0.00 B	0.10 ± 0.00 b

Values within a column followed by the same letters do not differ significantly according to ANOVA followed by Tukey's test (capital letters) or Kruskal-Wallis followed by Wilcoxon test (lower-case letters) at p ≤ 0.05. Data are expressed as mean ± standard deviation (SD).

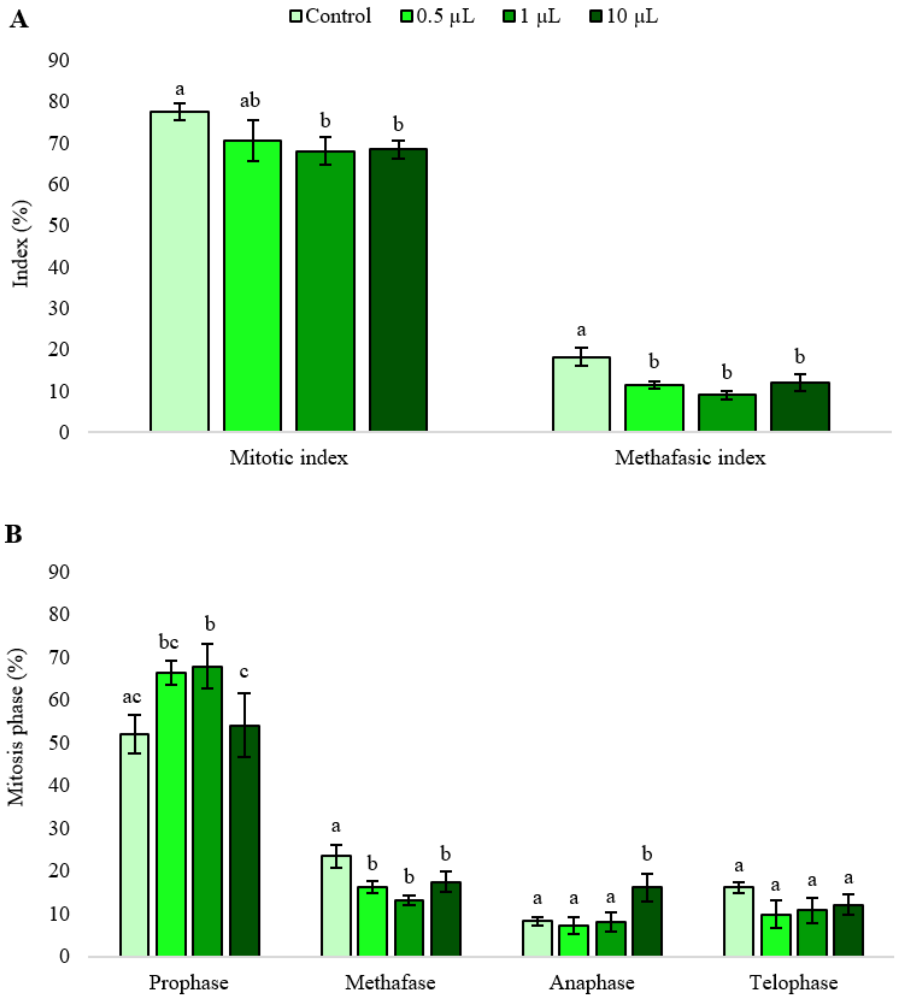

Figure 1. Mitotic and metaphasic indexes (a) and mitosis phases (b) of *Lactuca sativa* (lettuce) meristematic root cells exposed to different quantities of *Rosmarinus officinalis* (rosemary) essential oil. Bars with the same letter do not differ significantly according to ANOVA followed by Tukey's test (p ≤ 0.05).

Discussion

In this study, rosemary essential oil was shown to inhibit germination and seedling growth of the crop species *L. sativa* and the weed *E. plana*. This is the first documentation of the phytotoxic activity of *R. officinalis* essential oil on *E. plana*. Moreover, the essential oil interfered with cell division in the root meristem of *L. sativa*.

Considering the phytotoxic activity of natural products, it is relevant that the potential natural herbicides show species-specific effects, interfering in the physiology of weeds without harming crops of economic interest. The present study demonstrated that rosemary essential oil volatilizing into the airspace of the Petri dishes caused phytotoxic effects on the germination of *L. sativa* and *E. plana*, consistent with findings from studies employing the same methodology. Angelini et al. (2003), adding Tween 20 as a surfactant, observed that rosemary essential oil (also 1,8-cineole chemotype) completely inhibited the germination of three weed species (*Chenopodium album* L., *Echinochloa crus-galli* (L.) Beauv., and *Portulaca oleracea* L.) as well as *L. sativa*. The oil also significantly decreased the germination percentage of radish (*Raphanus sativus* L.) but did not interfere with pepper (*Capsicum annuum* L.) germination. Furthermore, rosemary essential oil (1,8-cineole/camphor chemotype) was less inhibitory to the germination of wheat (*Triticum aestivum* L.) compared to weeds that invade wheat plantations (*Avena sterilis* L. and *Sinapsis arvensis* L.) (Atak et al., 2016). The selective effect of rosemary essential oil observed in these studies suggests the need for further research to investigate its effects on desirable species found in pastures or crops affected by *E. plana* invasion.

Rosemary essential oil also interfered with the seedling growth of *L. sativa* and *E. plana*. Similarly, Chen et al. (2013), employing a different methodological approach (volatilization from fresh leaves), observed that rosemary volatiles significantly decreased the seedling growth of the weeds *Eleusine indica* (L.) Gaertn., *Cynodon dactylon* (L.) Pers., and *Digitaria sanguinalis* (L.) Scop.. A formulation of rosemary essential oil (1,8-cineole chemotype) affected germination and seedling growth of *Trifolium incarnatum* L., *Silybum marianum* (L.) Gaertn., and *Phalaris minor* Retz., and exhibited post-emergence effects when sprayed on these weeds under greenhouse conditions (Kaab et al., 2019). This demonstrates the strong potential for utilizing this chemotype of rosemary essential oil in weed control.

In this study, the phytotoxic effects observed on root length can be attributed to a mito-depressive effect of rosemary volatiles on *L. sativa* root tips. Root growth occurs by meristematic activity, resulting in an increase in the number of cells and subsequent elongation of them. Effects of allelochemicals on growth and development of exposed organisms can be related to a reduction in mitotic indexes (Graña, 2018). The reduction in cell activity could be due to changes in the duration of the cell cycle. Rosemary essential oil interfered with the mitotic process, as evidenced by a reduced mitotic index, but did not prevent root cells from initiating cell division, as low quantities of the essential oil increased the number of cells in prophase. However, volatiles interfered with the progression of the mitotic cycle, as observed with the arrested cells in anaphase in the highest quantity of essential oil. The cytotoxic activity observed in this study is consistent with studies using another model plant. Stojanović-Radić et al. (2010) observed that rosemary essential oil affected the mitotic process of *Allium cepa* L., significantly slowing down mitosis.

A high proportion of oxygenated monoterpenes and oxygenated sesquiterpenes in essential oils has been associated with a high level of phytotoxicity (Abd-ElGawad et al., 2021). Phytotoxic effects of complex substances, such as essential oils, have been commonly explained by action of their major compounds. Among rosemary essential oil constituents, 1,8-cineole, an oxygenated monoterpene, has been reported as a potent allelochemical (Abd-ElGawad et al., 2021; Miranda et al., 2014; Romagni et al., 2000; Silva et al., 2021). Furthermore, Romagni et al. (2000) showed that 1,8-cineole interfered with the mitotic index of *A. cepa*, severely decreasing all stages of mitosis. Silva et al. (2021) showed that phytotoxic effects of *Eucalyptus saligna* Sm. essential oil on germination and growth of *E. plana* were mainly related to 1,8-cineole, utilizing the same methodology and target species as presented here. Thus, 1,8-cineole might, at least partially, elucidate the effects of rosemary essential oil.

The results demonstrated the antiproliferative effect of rosemary essential oil on *L. sativa* root meristem. Cytogenetic analysis using plants as test systems is not only employed for evaluating cytotoxicity but also for predicting antitumor effect (Olaru et al., 2019) and pesticide activity (Stojanović-Radić et al., 2010). Further studies validating the predictive capacity of *L. sativa* are necessary to confirm the antitumor and pesticide potential of rosemary essential oil.

Essential oils have been investigated for their potential use in weed management as alternatives to synthetic herbicides (Raveau et al., 2020; Verdeguer et al., 2020; Werrie et al., 2020). Nanoencapsulation presents a promising opportunity in the development of multifunctional nanomaterials for more efficient release of agrochemicals and targeted delivery of pesticides, biological materials, and bioactive molecules (Rodrigues et al., 2017). The encapsulation of essential oils in starch can be achieved with a high level of efficiency and solubility in water. Encapsulated essential oils can serve as bioherbicides, slowly releasing into the soil due to their encapsulation (Alipour et al., 2019). This study demonstrated that even very small amounts of rosemary essential oil are highly phytotoxic. Therefore, further studies on the bioherbicide potential of rosemary essential oil should consider exploring the option of nanoencapsulation.

Conclusion

The high phytotoxic activity observed suggests that rosemary essential oil could serve as a potential bioherbicide. The results obtained from the cytogenetic evaluation (i.e., reduction in mitotic and metaphasic indexes) are consistent with those obtained for *L. sativa* root growth, wherein initial development was observed in all treatments, but subsequent root growth was inhibited. This confirms that *R. officinalis* volatiles inhibited root growth by delaying cell division.

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