A nanoemulsion of *Rosmarinus officinalis* L. essential oil with allelopathic effect against *Lactuca sativa* L. seeds

Uma nanoemulsão a partir do óleo essencial de *Rosmarinus officinalis* L com efeito alelopático em sementes de *Lactuca sativa* L

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ABSTRACT

Plant's essential oils have a wide range of allelopathic effects with potential uses as bioherbicides. In addition, the application of oils through nanoemulsions represents a promising alternative for agriculture, as it offers better performance and lowers toxic waste generation. Therefore, this study aimed to evaluate the chemical constitution of *Rosmarinus officinalis* (rosemary) essential oil, as well as its effects on germination, initial growth, Catalase (CAT), Peroxidase (POX), and Superoxide-Dismutase (SOD) enzymes activity of *Lactuca sativa* seeds. Nanoemulsions were produced at 5% concentration and then diluted with distilled water to 5.0, 7.0, and 10.0 mg/mL. We compared data obtained using variance (ANOVA) analysis, followed by Tukey's test at 5% probability. Rosemary oil showed a wide variety of terpenoid compounds, mainly the 1,8-cineol monoterpene, which accounted for 46% of the sample. The oil showed a dose-dependent negative allelopathic influence on all variables analyzed, causing a drop in germination percentage (%G), germination speed index (GVI), mean germination time (MTG), and leaf and root length. There was no change in CAT and SOD activity. The POX activity showed a reduction starting at the concentration of 7.0 mg/mL. The results showed allelopathic effects of rosemary oil, with potential use as a natural bioherbicide.

Keywords: Antioxidant activity, Gas chromatography, Monoterpenes, Rosemary.

ABSTRACT

Os óleos essenciais das plantas têm uma ampla gama de efeitos alelopáticos com usos potenciais como bioherbicidas. Além disso, a aplicação de óleos através de nanoemulsões representa uma alternativa promissora para a agricultura, pois oferece melhor desempenho e reduz a geração de resíduos tóxicos. Portanto, este estudo visou avaliar a constituição química do óleo essencial de Rosmarinus officinalis (alecrim), bem como seus efeitos na germinação, crescimento inicial, Catalase (CAT), Peroxidase (POX) e Superoxide-Dismutase (SOD), atividade enzimática das sementes de Lactuca sativa. As nanoemulsões foram produzidas a 5% de concentração e depois diluídas com água destilada a 5,0, 7,0 e 10,0 mg/mL. Comparamos os dados obtidos usando análise de variância (ANOVA), seguida pelo teste de Tukey com 5% de probabilidade. O óleo de alecrim mostrou uma grande variedade de compostos terpenóides, principalmente o monoterpeno de 1,8 cineol, que representou 46% da amostra. O óleo mostrou uma influência aleloopática negativa dose-dependente em todas as variáveis analisadas, causando uma queda na porcentagem de germinação (%G), índice de velocidade de germinação (GVI), tempo médio de germinação (MTG), e comprimento das folhas e raízes. Não houve mudança na atividade CAT e SOD. A atividade POX mostrou uma redução a partir da concentração de 7,0 mg/mL. Os resultados mostraram efeitos alelopáticos do óleo de alecrim, com uso potencial como bioferbicida natural.

Palavras-chave: Atividade antioxidante, Cromatografia gasosa, Monoterpenos, Alecrim.

1 INTRODUCTION

Nanotechnology is a science with applications in various productive sectors, such as cosmetics and health. However, its application in agriculture is recent and used as nano-fertilizers, nano-pesticides, nano-biosensors, and nanomaterials for soil remediation. Thus, nanotechnology can bring high yield to crops and healthy food production and is considered a revolution. In agricultural production, nanotechnology processes act as a tool to reduce costs, energy, inputs, and environmental impacts (Acharya & Pal 2020; Pérez-de-Luque 2020). Pest infestations control is another nanotechnology application that ensures the protection of the active substance, increases the solubility of fat-soluble actives in water, and better actives penetration in plant cells (Lima *et al.*, 2021).

Among the various nanotechnology applications in agriculture, an increasing alternative is nano-dispersion from plant actives to replace synthetic pesticides, which are very toxic and negatively affect the local ecosystem, causing enormous damage to the environment and human health. Therefore, essential oils have become one of the most studied plant derivatives for prospecting new pesticides of natural origin, having wide use in preparing nanostructured technological products for agricultural sector application (Chaudhari *et al.*, 2021; Gahukar and Das, 2020).

Essential oils are complex mixtures of lipophilic and volatile substances composed mainly of monoterpenes, sesquiterpenes, and phenylpropanoids compounds. They play an essential role in plant defense and their interaction with the environment (Campolo *et al.*, 2020). Among such interactions, the allelopathic effect is the direct interference that can stimulate and/or inhibit the development of other plant species

(Almeida *et al.*, 2019; Barbosa *et al.*, 2018; Campolo *et al.*, 2020; Karalija *et al.*, 2020; Matias *et al.*, 2017; Mirmostafaee, Azizi and Fujii, 2020; Salomão, Ferro and Ruas, 2020; Schandry and Becker, 2020; Takao, Imatomi and Gualtieri, 2015; Zucareli *et al.*, 2019).

Rosmarinus officinalis L. (Lamiaceae) species, produces an aromatic essential oil with many properties such as bactericidal, fungicidal, anti-inflammatory and analgesic and is worldwide cultivated (Borges, Lima, *et al.*, 2018; Santos Rodrigues *et al.*, 2020). In addition, its essential oil showed allelopathic activity in in vitro assays. However, essential oils are lipophilic and have low solubility in water-based products, which is a significant problem for developing new biopesticides (Alipour *et al.*, 2019; Donsì and Ferrari, 2016).

Nanoemulsions are colloidal systems formed by two immiscible liquids. They are stabilized by emulsifying substances (surfactants) with drop sizes ranging from 20 to 200 nm, improving water solubility. This process increases the biological activity of the capsulated compounds by increasing the contact surface, facilitating absorption, and constitute a viable alternative use of essential oils (Ahmad *et al.*, 2014; Lima *et al.*, 2021; Ortiz-Zamora *et al.*, 2020; Yukuyama *et al.*, 2016). Nanoformulations from rosemary essential oil have many activities. Among them are larvicidal (Duarte et al. 2015), anti-inflammatory (Borges et al. 2018a; Borges et al. 2018), antibacterial (Ghaderi et al. 2020), dyslipidemic (Santos et al. 2020), and osteoarthritis (Mohammadifar et al. 2021).

This work aimed to evaluate the allelopathic potential of a standardized nanoemulsion of *Rosmarinus officinalis* oil on the germination and initial growth of *Lactuca sativa* seeds.

2 MATERIAL AND METHODS

Experiments were carried out at Seeds and Forest Ecophysiology Laboratory (LASEF), at Federal University of Espírito Santo (UFES), Goiabeiras Campus, Vitória, ES; Chemistry Laboratory, at Federal Institute of Espírito Santo (IFES), Vila Velha Campus, ES, and Pharmacy Laboratory at Federal University of Amapá (UNIFAP).

2.1 PREPARATION OF ROSEMARY ESSENTIAL OIL NANOEMULSIONS STANDARDS

Prof. Dr. Caio Pinho Fernandes (Laboratory of Phytopharmaceutical Nanobiotechnology of the Pharmacy Course of the Federal University of Amapá) provided standard rosemary essential oil nanoemulsion samples. They were obtained through a low energy input method (Duarte *et al.*, 2015; Ostertag, Weiss and McClements, 2012). Samples composition was: 5% (w/w) polysorbate 20, 5% (w/w) essential oil extracted from rosemary leaves by hydrodistillation, and 90% (w/w) water, for a final mass of 50 g. The essential oil and polysorbate 20 (Vetec) were mixed under magnetic stirring (Marconi) for 30 min, followed by the addition of water and remaining under stirring for another 60 min. The stability data of the nanoemulsion was already established, validated, and standardized, according to Duarte et al., 2015.

For the experiment, the nanoemulsion with 5% of the essential oil (NANO) was diluted with distilled water to obtain the concentrations of 5.0 mg/mL, 7.0 mg/ml, and 10.0 mg/mL of *Rosmarinus officinalis* essential oil. Was used the same procedure to prepare a nanoemulsion without the essential oil, which served as a negative control (NC), to verify possible components interferences used to prepare the nanoemulsion. In addition, we inserted a distilled water-based blank (B) in the experiments.

2.2 ROSEMARY ESSENTIAL OIL CHEMICAL IDENTIFICATION BY GAS CHROMATOGRAPHY COUPLED TO MASS SPECTRUM (GC/MS)

Rosemary essential oil was submitted to chemical analysis using a Shimadzu GC-plus 2010 gas chromatography coupled to a 70eV electron impact mass spectrometry detector. Used DB-5MS column (30 m-0.25 mm-0.25 µm) and helium gas as the carrier in split mode at 1:20 ratio in the following parameters: injector at a temperature of 250°C, as well as the detector in this same condition. The heating condition of the chromatograph oven was initially at 50ºC for 1 minute, with a heating ramp varying at 5ºC per minute until the final temperature of 250ºC. The chemical components were identified by comparing the mass spectra from the NIST 5.0 database and the Kovats retention indices (IK) using an n-alkane standard (Adams, 2017).

2.3 GERMINATION BIOASSAY ON SEEDS OF *Lactuca sativa* L

The *L. sativa* seeds were used for the seed germination experiments (American variety great lakes 659 - Lechuga mesa 659), purchased commercially. For the experiments, we followed the methodology of MAPA, 2009 and Frazão & Silva, 2020. Was verified the biological potential of nanoemulsion at concentrations of 5.0, 7.0, and 10.0 mg/mL of essential oil of *R. officinalis* through the evaluation of germination and initial growth of seedlings, using the following parameters: Germination Index (GI) (GI = (N/A) x 100, where: N = number of germinated seeds and A = number of seeds in the sample); Allelopathy Index (AI) (AI= (G witness - G treatment) x 100 / G witness, where G stands for germination); Germination Velocity Index (GVI) (GVI = Σ (ni/ti), where ni and ti are the values of germinated seeds by the time of the experiment) and Mean Germination Time (MGT) MGT = (Σ ni.it)/Σ ni, where ni = number of seeds germinated per day and it = incubation time) of *L. sativa* according to Labouriau & Viladares 1976 and Maguire 1962. For germination tests, were sowed 20 seeds per Petri dish lined with a double layer of filter paper (Unifil) moistened with 2.5 mL of each nanoemulsion at concentrations of 5.0, 7.0, and 10.0 mg/mL of essential oil of *R. officinalis* (NANO). Were used five repetitions per treatment. The components were diluted that make up the nanoemulsion production without rosemary essential oil (CN) in the same proportions as for the NANO for negative controls. Control treatment was distilled water (B). Plates were kept in a germination chamber (BOD) under constant light at 20ºC for seven days. The monitoring of germinated seeds was every 24 hours. All experiments were performed in triplicate.

2.4 CATALASE, PEROXIDASE, AND SUPEROXIDE DISMUTASE ENZYMES ACTIVITY

Were macerated 0.3g of lettuce seeds to obtain the crude enzymatic extracts. The macerate was diluted to determine catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) activities. Seeds were previously submitted to germination with the rosemary nanoemulsions at concentrations of 7 mg/mL and 10 mg/mL for 24 hours at 20ºC in liquid N_2 because these concentrations were the most active in the germination test. For the extraction, plant material was homogenized with 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM Na_2EDTA and 1% (w/v) polyvinylpolypyrrolidone (PVPP). For the extractions, was used mortar and pestle with liquid nitrogen. The homogenate was centrifuged at 12000 xg for 15 min at 4 °C and used the supernatant for the enzyme activity assays.

The SOD activity was based on Beauchamp & Fridovich 1971, Giannopolitis & Ries 1977 and Del Longo et al 1993, and determined by adding 50 µL of crude enzyme extract to 2.95 mL of a reaction medium consisting of 100 mM sodium phosphate buffer, pH 7.8, containing 50 mM methionine, 1 mM *p*-nitrotetrazolium blue (NBT), 5 mM EDTA, and 10 mM riboflavin. The reaction took place at 25°C in a reaction chamber under illumination from a 15 W fluorescent lamp kept inside a closed box. After 5 min of light exposure, the lighting was turned off, and measured the blue formazan produced by the photoreduction of NBT at 560 nm. The absorbance at 560 nm of a reaction medium precisely like the previous one, but kept in the dark for the same time, served as a "blank" and was subtracted from the sample reading that received illumination. One unit of SOD was defined as the amount of the enzyme required to inhibit 50% of the photoreduction of NBT.

The POX activity measurement was based on Chance & Maehly 1955 Kar & Mishra 1976. Were added 50µL aliquots of the enzyme extract to 2.95 mL of a reaction mixture consisting of 100mM potassium phosphate buffer, pH 6.8, 20 mM pyrogallol, and 20 mM H_2O_2. We measured the increase in absorbance at 420nm (Kasuaki, IL-593) at 25ºC through purpurogalin production. POX activity was determined based on the slope of the straight line after the beginning of the reaction. The enzymatic activity was calculated using the molar extinction coefficient of 2.47 mM cm^{-1}, and the result was in µmol $min^{-1} \cdot mg^{-1}$ of protein. Were used four replicates with duplicates.

CAT activity followed the protocol of Anderson et al 1995 and Havir & McHale 1987, in which we added 50 µL of the crude enzyme extract to 2.95 mL of a reaction medium, which consisted of 100 mM potassium phosphate buffer, pH 6.8, and 12.5 mM H_2O_2. Was measured the decrease in absorbance (Kasuaki, IL-593) at 240 nm at 25°C during the first minute of the reaction. CAT activity was determined based on the slope of the straight line after the beginning of the reaction. The enzyme activity was calculated using the molar extinction coefficient of $36M.cm^{-1}$, and the result expressed as $\mu mol.min^{-1} \cdot mg^{-1}$ of protein. Were used three repetitions with duplicates in an entirely randomized design (DIC) in all analyses.

2.5 STATISTICAL ANALYSIS

The data were submitted to variance analysis, and if the treatments effects treatments showed a significant difference ($p<0.05$), we compared the means using Tukey's test (Sisvar 5.6).

3 RESULTS AND DISCUSSION

3.1 COMPOUNDS IDENTIFIED FROM THE ROSEMARY ESSENTIAL OIL BY GC/MS

In the GC/MS analysis, a total of 15 substances consisting mainly of monoterpenes could be identified, with 1,8 cineol (46.47%), Thujanol (18.21%), and α - pinene (12.42%) found in the highest percentage (Table 1).

Table 1 - Characterization of chemical composition of *Rosmarinus officinalis* essential oil.

Signal	**RT***	**Identified Substance**	**Concentration (%)**	**Kovats Index (IK)**
1	5.025	α-Pinene	12.42	939
2	5.394	α-Fenchene	1.78	951
3	6.124	*β*-Pinene	7.63	980
4	6.454	Myrcene	0.52	991
5	7.507	Cimene	0.97	1022
6	7.645	Limonene	3.02	1031
7	7.752	1,8-Cineole	46.47	1033
8	8.644	*γ*-Terpinene	0.22	1062
9	10.100	Linalool	1.01	1098
10	11.870	Thujanol	18.21	1146
11	12.730	Borneol	1.43	1165
12	13.184	Terpin-4-ol	0.27	1177
13	13.738	α-Terpineol	1.01	1189
14	17.714	-	0.33	-
15	23.226	α-Santalene	4.71	1420
Total			100	

*TR=Retention Time

Monoterpene's presence in essential oils is widely reported in several studies as the main metabolites. They are responsible for several biological activities, including the allelopathic effect (Souza Filho et al. 2009; El Sawi et al. 2019; Assaeed et al. 2020). The chemical composition of the essential oil of *R. officinalis* can vary according to many factors. Some of them are extraction conditions, vegetative stage, agrochemicals used, storage, and climatic conditions. Noteworthy in its composition are 1,8 cineol, camphor, and α-pinene, in addition to borneol, limonene, and α-terpineol in smaller amounts (Andrade et al. 2018; Borges et al. 2019; Karalija et al. 2020). Variation in the chemical composition of the essential oil can interfere with herbicidal activity. El Mahdi et al. 2020 found differences in *Rosmarinus officinalis* oil chemotypes activity in pre-and post-germination treatments on *Amaranthus retroflexus* L. and *Lolium perenne* L seeds. The higher camphor concentration oil showed higher activity than oils with α-pinene/1,8-cineole, α-pinene, and α-pinene/1,8-cineole/camphor. Such chemical variations have been observed in essential oils with a higher percentage of 1,8-cineole (Kabouche et al. 2005; Machado et al. 2013; Maia et al. 2014; Miranda et al. 2015), as well as intermediate amounts compared to the other compounds in the oil (Boix et al. 2010; Ribeiro et al. 2012). Due to this diversity in the concentration of significant components of essential oils, it is crucial always to perform their chemical characterization because it can interfere in the germination and development of plants (Weir et al 2004).

3.2 *ROSMARINUS OFFICINALIS* NANOEMULSIONS EFFECTS ON GERMINATION TESTS

Rosemary essential oil nanoemulsion showed effects on germination. The 10.0 mg/mL concentration showed the most significant allelopathic effect on all variables analyzed (Table 2).

Table 2 - Germination Index (GI), Allelopathy Index (AI), Germination Speed Index (GVI), and Mean Germination Time (MTG) of *L. sativa* seeds subjected to different concentrations of rosemary nanoemulsion compared to the controls.

Nanoemulsion *R. officinalis*	**Essential oil Concentrationmg /mL**	**%G**	**AI (%)**	**GVI**	**MTG**
Nano	5.0	69 a	16.87	20.42 a	3.75 a
	7.0	60 b	27.71	13.45 a	4.40 a
	10.0	39 b	53.00	7.21 c	5.59 c
B	-	83 a	-	25.5 a	3.56 a
NC	5.0*	86 a	-3.61	63.51 b	1.90 b
	7.0*	84 a	-1.20	71.36 b	1.28 b
	10.0*	84 a	-1.20	65.7 b	1.45 b

Nano = nanoemulsion with essential oil, B = blank (distilled water), NC = negative control (*nanoemulsion without essential oil diluted in the same way as the nanoemulsion with oil). Averages followed by the same letter did not show statistical differences from each other by Tukey test at 5% probability.

Only 39% of lettuce seeds germinated at the 10.0 mg/mL concentration. This same concentration showed the lowest germination speed when compared to the other concentrations. Consequently, there was an increase in the AI and average germination time compared to the negative control and the blank. There was no significant difference in allelopathic activity at the lowest concentration, and the 7.0 mg/mL concentration showed a 60% decrease in lettuce seed germination. These results indicate allelopathic activity in rosemary essential oil nanoemulsion. The higher is the nanoemulsion concentration, the higher is the activity, which may cause physiological damage in the cells.

The results obtained showed that rosemary essential oil presents an allelopathic effect. Thus, it can be an alternative for products with herbicidal activity. The essential oil of *R. officinalis* has demonstrated its phytotoxic activity against weed seeds and little activity in the seeds such *Cucumis sativus* L. and *Solanum lycopersicum* L, valuable species to agriculture (Ibáñez & Blázquez 2020). The present study obtained similar results to other studies on the effects of rosemary essential oil on the inhibition of germination of different seeds of species such as weeds or lettuce, which is considered a model species in this type of study (Hillen et al., 2012; Rahimi et al. 2015; El Mahdi et al. 2020; Mirmostafaee et al. 2020; Verdeguer et al. 2020).

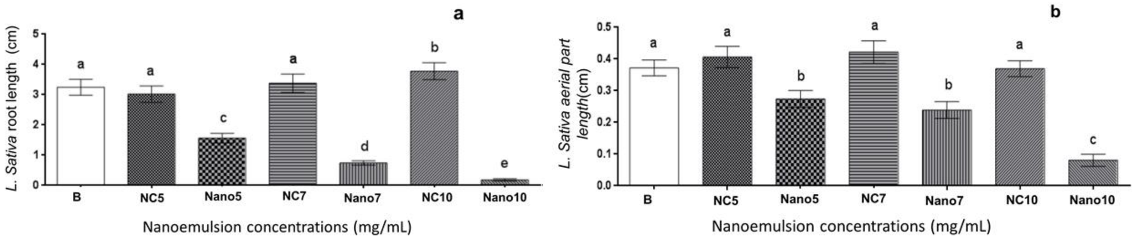
The way the essential oil is conveyed in the experimental protocols can vary significantly because the hydrophilic character of the experiments makes it difficult to solubilize and dilute the oil in water. For this purpose, solutions are made with the oils to facilitate this solubilization. The use of solvents and surfactant can facilitate incorporating the oil into this aqueous system of the experiment (El Mahdi et al. 2020). Using nanostructured formulations as a medium for the essential oil is an alternative to minimize experimental errors. It facilitates the availability of the actives in the plant cells, thus increasing the allelopathic effect.

Rosemary nanoemulsion can be a stable product for farmers because of the ease of transport, solubility, and oil dilution (Ostertag et al. 2012; Duarte et al. 2015). Studies on preparations of nanostructures with essential oils are scarce. Several research has been showing its various applications, such as preserving stored foods by its pesticide action of oils, increasing the shelf life of foods by its protection against fungi and bacteria, and in agriculture, in weed control (Usman et al. 2020). It is possible by the ease of nanoformulation dispersed products being readily soluble in water, enhanced bioefficacy, and controlled release (Chaudhari et al. 2021). To date, only one study with nano encapsulated *Rosmarinus* sp essential oil has been presented with effect on germination in seeds of *Amaranthus retroflexus* and *Raphanus sativus*, allowing for the broad range of action with nanostructured products (Alipour et al. 2019).

The increase in GVI observed in the negative control probably occurs due to polysorbate, which can decrease the surface tension of water, facilitating its absorption by the seed, showing that the allelopathic effect was caused by the allelochemicals of the essential oil, once the nanoemulsion raises the allelochemicals bioavailability, allowing solubilization of hydrophobic compounds present in the essential oil (Ben Jemaa et al. 2019).

There was a significant growth reduction in the aerial part and root of lettuce seedlings submitted to all concentrations of the tested rosemary nanoemulsions. This influence was dose-dependent with the concentration of 10.0 mg/mL (Figure 1).

Figure 1 - Root length (**a**) and aerial part length (**b**) of lettuce seedlings, submitted to different concentrations of rosemary essential oil nanoemulsions. **B** = blank (distilled water), **NC** = negative control (nanoemulsion without essential oil at dilutions of 5.0, 7.0 and 10.0 mg/mL), **Nano** = nanoemulsion with essential oil (5.0, 7.0 and 10.0 mg/mL).



Such effects can be because of the specific action of its main chemical constituents or, possibly, by the interaction among them. Monoterpenes have great inhibitory potential, causing morphological and physiological alterations. They affect plant cells such as membrane permeability, cell division, photosynthesis, respiration, transpiration, microtubules, cell growth (Reis et al. 1992; Rosado et al. 2009; Miranda et al. 2014). There are reports of phytotoxic activities related to the main monoterpene identified in this work. The 1,8-cineole can affect germination and development of several plant species, such as *Chenopodium album*, *Portulaca oleracea*, *Echinochloa crus-Galli*, *Raphanus sativus*, and *Lactuca sativa* (Ismail et al. 2013; Verdeguer et al. 2020), as well as *Ailanthus altissima* (Karalija *et al.*, 2020).

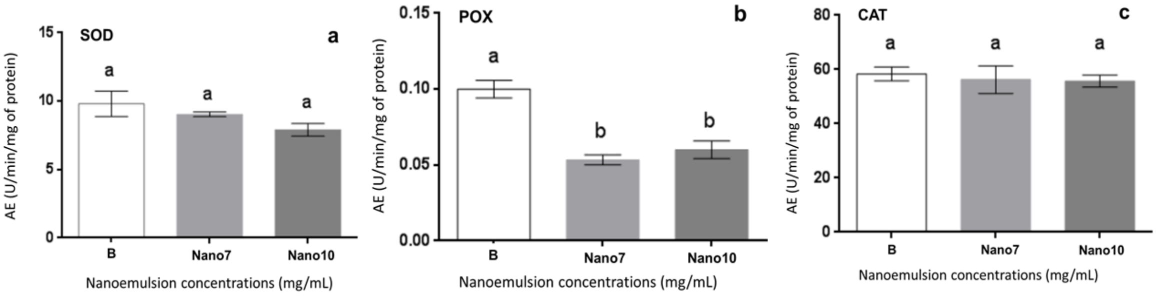
3.3 *ROSMARINUS OFFICINALIS* NANOEMULSIONS EFFECTS ON ENZYME ASSAYS

Rosemary nanoemulsion allelochemicals can affect plants by inducing oxidative stress by excessive reactive oxygen species (ROSs) (Alipour et al. 2019). Among the main ones produced, hydrogen peroxide (H_2O_2), superoxide radical, and singlet oxygen stand out. These are very unstable compounds, and when in excess, they exert direct oxidizing action or act as signaling pathways in cell degradation, impairing vital physiological processes such as germination and early growth (Anjum et al. 2015). Other specific effects are attributed to terpenes, which are present to a large extent in rosemary. Due to their lipophilic character can directly damage lipid structures, culminating in increased oxidation levels (Singh et al. 2006). In response, plants protect themselves from the damage caused by increased oxidative stress mainly through enzymatic mechanisms and, to a lesser extent, non-enzymatic ones (Barbosa *et al.*, 2014; Mittler, 2002).

Superoxide ion dismutases (SODs) and H_2O_2 removing enzymes (Catalase and Peroxidases) are some of the enzymes involved in this process (Usman et al. 2020). SODs are metalloenzymes, and they are considered a first-line defense against SROs, promoting superoxide ion (O_2^-) dismutation into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Mittler 2002; Nascimento et al. 2020; Chaudhari et al. 2021). Catalase (CAT) and Peroxidases (POX) act by removing the H_2O_2 generated by ORSs and also by other metabolic pathways, and thus act in a synchronized manner, ensuring greater tolerance to oxidative stress (Gill & Tuteja 2010; Verdeguer et al. 2020). Therefore, alterations in the activity of these antioxidant enzymes may indicate losses to the detoxification process with consequent damage to plant development (Barbosa et al. 2010; Nascimento et al. 2020).

We did not observe significant changes in SOD and CAT activity in this work, and POX activity reduced from 7.0 mg/mL concentration in treatments with rosemary nanoemulsions (Figure 2).

Figure 2: Rosemary essential oil nanoemulsions effects at 7.0 mg/mL and 10.0 mg/mL concentrations on the activity of the enzymes superoxide dismutase (SOD) (a), peroxidase (POX) - (b), and catalase (CAT) - (c). B = blank (distilled water), Nano - Nanoemulsion. Means followed by the same letter are not statistically different from each other by Tukey's test at 5% probability.



Alipour et al. 2019 studied the effects of rosemary oil also in its encapsulated form and found elevated oxidative stress in radish and amaranth plants. Scognamiglio et al. 2013 observed other oxidative effects in lettuce specimens submitted to contact with the oils of plants of the genus *Lavender* and *Thymus*, known to have a terpenoid composition close to that found in rosemary. The monoterpene 1,8-cineole and α-pinene, more specifically, main components of rosemary oil, showed strong oxidative potential in corn plants and increased CAT, POXs, and SOD enzymes activity and oxidative stress in

Cassia occidentalis plants (Zygadlo & Zunino 2004; Singh et al. 2006). However, in this study, SOD and CAT enzymes activity remained unchanged in all treatments.

These results may indicate that there was no elevation of ROS levels and oxidative stress, or maybe that the *L. sativa* species has non-enzymatic mechanisms and the expression of other enzymes to cope with the increased toxicity. Jucoski 2011 reported these results in *Eugenia uniflora* plants, which showed elevated ascorbate levels when exposed to high levels of ROS. The *C. occidentalis* showed up to a thirteen-fold increase in glutathione reductase enzyme activity when exposed to high doses of monoterpenes (Singh et al. 2006).

POX enzyme, in turn, showed a drop of approximately 50% in its activity from the concentration of 7.0 mg/mL. The explanation for the POX enzyme activity fall can be the stress generated by rosemary oil, which did not cause an increase in the levels of H_2O_2. We did not observe a change in peroxide levels after quantifying the peroxide content in corn plants grown in contact with a solution rich in the monoterpene 1,8 cineol (Zygadlo & Zunino 2004). In addition, the CAT enzyme showed stability in its activity (Figure 2), which seems to indicate the presence of other metabolites involved in the oxidation process and the damage observed in the germination and growth of *L. sativa.*

In addition, possibly some chemical component present in the oil or even the interaction between them is related to the reduction and/or inhibition of the POX enzyme activity, and consequently, the lower vegetative growth of the plant. POX, besides its role in cell protection against oxidative damage, can also act as a signaling agent in plant development, modulating the process of cell elongation and hypocotyl growth through the accumulation of OH^-, a critical agent in the formation of lignin (Maia et al. 2012). Thus, the decrease in POX activity observed in this work may have impacted the reduction of initial seedling growth, as observed for lettuce seeds submitted to extracts of thyrrhiza bulb (Muniz et al. 2007).

4 CONCLUSION

Both seed germination and initial growth of lettuce were negatively affected by rosemary essential oil nanoemulsion. This allelopathic effect varied with the concentration and chemical constituents of the oil. Results obtained in this study allow suggesting the sustainable use of rosemary essential oil nanoemulsion as a bioherbicide in agriculture.

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