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ASSESSMENT OF THE BIOLOGICAL INFLUENCE OF NATURAL EMULSIFIER ON THE GROWTH AND INITIAL DEVELOPMENT OF *Sinapis alba* L. AND *Lactuca sativa* L. IN LABORATORY CONDITIONS

SUMMARY

Under controlled laboratory conditions, the biological influence of the emulsifiers Polysorbate 80, Glyceryl Monostearate SE, and Olivem 1000 on seed germination and initial development of *Sinapis alba* L. and *Lactuca sativa* L. was evaluated and compared. It was found that the emulsifiers included in the study and the applied concentrations had an indifferent to lethal effect on seed germination and initial development of the target plants. It was found that the emulsifier Polysorbate 80 can be used in concentrations ranging from 0.05 to 0.2% v/v, while Glyceryl Monostearate SE and Olivem 1000 in concentrations not higher than 0.025% v/v when performing screening laboratory studies to establish the biocidal effect of essential oils. Further studies are needed, including validation of the obtained experimental results in laboratory and vascular trials, to establish the influence of emulsifiers in combined application with essential oils or hydrolates of plant biomass from plants with proven allelopathic potential.

Key words: emulsifiers, *Sinapis alba* L. and *Lactuca sativa* L. inhibition; allelopathy; Biotest.

INTRODUCTION

Being a natural creation on our planet, soil is the fundamental base of terrestrial ecosystems, serves as the base for forestry and agriculture, supports biodiversity, filters water, and stores carbon to slow down climate

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change. It is vital to life on Earth because it helps produce food, provides raw materials for products like fibre and medications, and plays a vital role in the carbon and nutrition cycles of the planet (Popović *et al.*, 2024; Miskoska Milevska *et al.*, 2025). Limiting the degree of weed infestation in conditions of modern intensive agriculture is a permanent, complicated problem, limited by a complex of multiple factors, especially after the growing concerns in society about the multifaceted negative impacts resulting from the massive use of modern synthetic herbicides in agricultural practice (Aktar *et al.,* 2009; Božović *et al.,* 2018; Rakašćan *et al*., 2021; Lakić *et al.,* 2022; Monteiro and Santos, 2022; Rajak *et al*., 2023; Zhou *et al*., 2024). Despite the fact that in recent decades herbicides have become established in industrial agricultural production as effective chemical substances for controlling weeds, their intensive and indiscriminate application, as the main means of limiting the degree of weed infestation in modern agriculture, on the one hand is a prerequisite for the generation of herbicide-resistant weed species. On the other hand, as a result of their mass implementation in agricultural practice, it is a prerequisite for the accumulation of residual amounts of chemical substances, which pose a serious threat to the environment and, respectively, to human health (Popović *et al*., 2013; Paul *et al*., 2024). The discovery and introduction of alternative solutions to synthetic herbicides is a priority of modern organic agriculture to limit the harmful impact of weeds with minimal anthropogenic impact on agrophytocenoses (Nikolova *et al.*, 2020; Nath *et al*., 2024; Parven *et al*., 2024).

Many scientific studies in the last decade have focused on the possibilities of applying essential oils as a promising alternative to modern herbicides (Scognamiglio *et al*., 2013; Amri *et al*., 2012; 2017; Nikolova and Berkov, 2018; Nikolova, 2021; Bellache *et al*., 2022). According to the summarized studies of Rehman *et al.* (2016), Bellache *et al*., (2022) the chemical composition of essential oils includes highly functionalized chemical classes, such as monoterpenoids, sesquiterpenoids and phenylpropanoids, with most of the studies being aimed at determining the inhibitory effect of essential oils on the germination and initial development of weed seeds (Amri *et al.,* 2017; Nikolova, 2021). When conducting screening studies to determine the inhibitory effect of essential oils on the germination and initial development of weed seeds in laboratory conditions, emulsifiers are added to facilitate and stabilize the mixing of the oils in water, thereby forming their uniform distribution in the working solution (homogeneous mixture) to form a stable emulsion (Fritz *et al.,* 2022).

According to Benchaa *et al.* (2018), Kong *et al*. (2021), Amri *et al.* (2017), Nikolova and Berkov (2018), Nikolova (2021), Bellache *et al.* (2022), Zheljazkov *et al.* (2021) Yaman *et al.* (2024), when performing screening laboratory studies with essential oils, the liquid emulsifier Polysorbate is widely used. According to Maher *et al.* (2023), polysorbate [(x)-sorbitan mono-9-octadecenoate poly(oxy-1.2-ethanediyl)] is a non-ionic surfactant obtained by esterification of sorbitol with fatty, stearic, lauric, oleic, and palmitic acids, commercially available as Poegasorb, Kolliphor PS, Alkest TW, Scattics, Canarcel, Poegasorb, Montanox, Tween, and Kotilen with different polysorbate numbers of 20, 40, 60 or 80, which determine the lengths of the fatty acid chain in them.

Despite the established methodological approaches in conducting screening laboratory studies to establish the influence of essential oils as an alternative means of limiting the degree of weed infestation and/or their use as allelopathic suppressive substances on the germination and initial development of weed seeds, there are sporadic reports on the biological influence of the emulsifiers used on the germination and initial development of the target plants (Fabri *et al.,* 2019; Terzić *et al.,* 2019; Han *et al.,* 2021; Kong *et al.,* 2021; Milunović *et al*., 2022; Wang *et al.,* 2022). The aim of this study was that under controlled laboratory conditions, to establish and compare the independent influence of the emulsifiers Polysorbate 80, Glyceryl Monostearate SE, and Olivem 1000 on the germination and initial development of *Sinapis alba* L. and *Lactuca sativa* L.

MATERIAL AND METHODS

Experimental design

The experimental work was carried out in 2024 in laboratory conditions at the Institute of Ornamental and Medicinal Plants - Sofia. *Lactuca sativa* L. variety "Great Lakes" and *Sinapis alba* L. - species with proven sensitivity to potentially toxic substances (Zloch *et al.,* 2018; Adamcová *et al*., 2019; Mardani *et al*., 2019; Vieira and Droste, 2019; Vaverková *et al*., 2020; Vieira *et al*., 2024) were used as target plants. Unlike *Lactuca sativa* L., *Sinapis alba* L. is a plant species with proven ecological stability and plasticity and high adaptability to adverse climatic conditions, biotic and abiotic stress (Muhammad et al., 2023), and is often used as a target plant species when performing screening studies in laboratory conditions (Banaszkiewicz and Wysocki, 2012; Adamcová *et al*., 2019; Sakaki and Nedvěd, 2023; Harrison *et al*., 2024).

Natural emulsifiers included in the study are presented in Table 1.; 1.6; 3.0; 6.0, and 12.00% v/v.

Table 1. Natural emulsifiers included in the study

№	Designation	Description
1.	Polysorbate 80	Nonionic surfactant obtained by ethoxylation of sorbitol with fatty acids
2.	Glyceryl Monostearate SE	Self-emulsifying non-ionic lipophilic emulsifier of vegetable origin. Obtained from palm oil, olive oil, sunflower oil, and stearic acid
3.	Olivem 1000	Self-emulsifying natural emulsifier, produced based on olive oil

All available natural emulsifiers were applied in the concentration range 0.00; 0.0125; 0.025; 0.05; 0.1; 0.2; 0.4; 0.8. Semi-solid 0.75% agar-agar was used as the medium for the development of the target plants. 40 ml of agar-agar were pipetted into Petri dishes (90 mm), and after the agar gel was formed, 10 seeds (Hamad *et al*., 2023) of *Lactuca sativa* L. and *Sinapis alba* L. were placed in each dish, and then they were placed in the dark in a thermostat for incubation at a temperature of 22 ± 2 °C for ten days. Agar-agar with distilled water was used as the control. To determine the influence of the emulsifiers included in the study on the germination and initial development of the target plants, indicators related to the germination and initial development of the target plants were recorded, and complex indices and coefficients were determined, presented in Table 2.

Table 2. Indicators for assessing seed germination and initial development of the target plants *Sinapis alba* L. and *Lactuca sativa* L.

Evaluation parameter - Author	Calculation	Explanation of the evaluation parameter
Percentage of germinated seeds, SG% Saxena et al. (1996)	$SG_{\%} = \left(\frac{NSG}{TNS}\right) * 100$	NSG - number of germinated seeds, TNS total number of seeds planted for the included variants and repetitions in the experiment.
Length of root, hypocotyl, and seedlings, cm	$SL = \sum_{i=1 \div 20}^{n} I/n$	I – number of individual measurements of plant organs for the included variants and repetitions in the experiment
Coefficient of allometry, CA Nasr&Mansour, 2005	$CA = \frac{L_h}{L_r}$	L_h - hypocotyl length, cm; L_r - root length, cm.
Median lethal concentration LC 50 Srinivasan (2004)	Probit analysis based on Finney's Method	%-Percentage of germinated seeds; Lh- hypocotyl length, cm; Lr - root length, cm; Ls - seedling length, cm; FB_h - fresh hypocotyl biomass, g; FBr - fresh root biomass, g; FBs-fresh seedling biomass, g.
Biological impact rank, Re Bashar et al. (2023)	$R_e = LC\ 50_{GP\%} + LC\ 50_{Lr\ cm/g} + LC_{Lh\ cm/g}$	
General Plant Growth Index, GI Gariglio et al. (2002).	$GI = \left[\left(\frac{G}{G_0}\right) \cdot \left(\frac{L}{L_0}\right)\right] . 100$	G and G_0– germinated seeds in the treated and the control variant (%); SL– seedling length in the treated variants; L_0– seedling length in control variant, taken as 100%.

The obtained experimental results were statistically processed with the software products Statgraphics Plus for Windows Ver. 2.1 and Statistica Ver. 10 through one-factor and three-factor analysis of variance (ANOVA) using LSD values, to establish the statistically significant influence, by Student-Fisher t-test at a confidence interval of 95% and error α = 0.05 as follows: Factor A (emulsifiers) a_1 - Polysorbate 80, a_2 - Glyceryl Monostearat SE and a_3 - Olivem

1000; Factor B (concentrations, % v/v) b_1 – 0.0, b_2 – 0.0125, b_3 – 0.025, b_4 – 0.05, b_5 – 0.1, b_6 – 0.2, b_7 – 0.4, b_8 – 0.8, b_9 – 1.6, b_{10} – 3.0, b_{11} – 6.0 and b_{12} – 12.0% v/v and Factor C (target plants) c_1 – *Sinapis alba* L. and c_2 – *Lactuca sativa* L. The percentage of germinated seeds (GP%) was transformed using arcsin (arcsin $\sqrt{x}$) (Ayeb-Zakhama and Harzallah-Skhiri, 2016). The strength of influence (weight of factors) was determined by η2 at a reliable factorial variance of 99% (Plohinskii, 1967).

RESULTS AND DISCUSSION

The natural emulsifiers included in the commercial formulations of the study had an indifferent to inhibitory effect on the laboratory germination (SG%) of the seeds of the target plants included in the study (*Lactuca sativa* L. and *Sinapis alba* L.), disproportionate to the increase in the concentration of the emulsifiers compared to the control variant with distilled water (Table 3).

From the analysis of the experimental results presented in Table 3, it is evident that significant differences are established in the biological influence of the natural emulsifiers included in the study on the germination of *L. sativa* and *S. alba* seeds under in vitro conditions. The application of Polysorbate 80 in concentrations from 0.4 to 12.0 % v/v has a statistically proven ($P=0.05$) inhibitory effect on the laboratory germination of the seeds of the target plant species included in the study, while when applying Glyceryl Monostearate SE and Olivem 1000 a statistically significant inhibitory effect on the studied indicator is established when using concentrations in the range from 0.05 to 12.0 % v/v. The inhibitory effect on the studied indicator under the influence of Polysorbate 80 is relatively more pronounced in *L. sativa* compared to the *S. alba* seeds included in the study. It was found that the inhibitory effect of Polysorbate 80 on laboratory seed germination in target plants is species specific and increases disproportionately with increasing its concentration in the agar gel. Therefore, concentrations in the range of 0.0125 ÷ 0.2% v/v of Polysorbate 80 can be used as an emulsifier for essential oils when performing screening laboratory studies in laboratory conditions, while Glyceryl Monostearate SE and Olivem 1000 should be used in significantly lower concentrations of 0.0125 to 0.025% v/v. An exception to the described dependence is found when applying the emulsifier Olivem 1000, which at the lowest applied concentration of 0.0125% v/v has a statistically proven ($P=0.05$) inhibitory effect on laboratory seed germination, but only in the target plant *L. sativa*.

From the two-factor analysis of variance performed to determine the weight of the factors (η^2) the hierarchical distribution of variation in terms of laboratory germination of the seeds of the species included in the study, the relatively largest share of the total variation is occupied by Factor B (concentrations) (η^2 = 71.9%), followed by Factor A Factor A (emulsifiers) η^2 = 7.9%.

Table 3. Influence of the included emulsifiers on the laboratory germination of the seeds of *Sinapis alba* L. and *Lactuca sativa* L.

Indicators	Emulsifiers					
	Polysorbate 80		**Glycerol Monostearat SE**		**Olivem 1000**	
Target plants / **Concentrations, %**	***S.alba***	***S.sativa***	***S. alba***	***S. sativa***	***S.alba***	***S. sativa***
0.0	67.9g	72.9h	67.9f	72.9h	67.9i	72.9g
0.0125	68.4g	71.6h	67.2f	67.2g	66.9hi	67.2f
0.025	68.9g	71.7h	57.4e	71.6h	63.6h	60.0e
0.05	67.0g	56.8g	29.3d	63.7fg	51.5g	56.9e
0.1	67.2g	56.8g	15.7c	56.9e	40.8f	56.8e
0.2	67.2g	56.8g	9.1b	60.0ef	33.4e	57.2d
0.4	56.9f	56.8g	0.0a	39.3d	26.7d	36.3c
0.8	40.0e	56.8g	0.0a	33.3c	18.4c	18.4b
1.6	27.4d	40.0f	0.0a	33.2c	9.1b	10.6a
3.0	18.5c	18.4e	0.0a	18.4b	0.0a	0.0a
6.0	10.8b	0.0a	0.0a	0.0a	0.0a	0.0a
12.0	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a

Independent action and interaction of the studied factors

Factor A (emulsifiers)	**Factor B (emulsifiers)**		**Factor C (emulsifiers)**	
a_1	b_1	70.32h	c_1	31.04a
a_2	b_2	66.5gh	c_2	42.50b
a_3	b_3	60.8g		
	b_4	52.3f		
	b_5	47.9f		
	b_6	45.8f		
	b_7	36.0e		
	b_8	27.8d		
	b_9	20.1c		
	b_{10}	12.0b		
	b_{11}	1.8a		
	b_{12}	0.0a		

Factors	MS	η^2
A	6535.7	7.9
B	10769.4	71.9
C	7093.2	4.3
AxB	356.2	4.8
AxC	3816.5	4.6
BxC	303.9	2.0
AxBxC	292.6	3.9
Error	6.3	0.6
The relationship between variable	506472.3	

Legend: a, b, c, d, e, f, g, h, i – LSD statistically significant differences at P=0.05. Values marked with the same letter are statistically insignificant at P=0.05; MS – mean sum of squares - ANOVA; η^2- strength of influence of factors in the factorial plane.

The values of the variances of the interaction of the studied factors determine the relatively smallest share of the total variation (η^2 varies in the range from 2.0 to 4.8%), although their influence is statistically significant at P=0.05.

In line with the established statistically significant differences in laboratory seed germination is the determined LC 50 of the emulsifiers included in the study, which can be ranked in the following ascending order: Polysorbate 80 → Glyceryl Monostearate SE → Olivem 1000, depending on the type of target plant (Table 4).

Table 4. LC 50 of the emulsifiers included in the study on laboratory seed germination.

LC %	**Emulsifiers**					
	Polysorbate 80		**Glycerol Monostearat SE**		**Olivem 1000**	
Target plants	***S. alba***	***S. sativa***	***S. alba***	***S. sativa***	***S. alba***	***S. sativa***
LC50	1.334	1.209	0.057	0.638	0.215	0.289
LC50LL	.157	0.869	0.050	0.493	0.178	0.235
LC50UL	1.539	1.682	0.064	0.826	0.258	0.354

Note: LL - lower Limit LC 50, UL – Upper Limit LC 50

Similar results were obtained in the experimental work of Gálvez *et al.* (2018), according to which the application of non-ionic surfactants (Triton X100, Brij 35, Tween 80, and Glucopone 600) to seeds of *Allium cepa* L. and *Lactuca sativa* L. has potential toxicity, expressed in inhibition of seed germination of the target plants included in the study, which requires them to be used in significantly lower concentrations - <0.65 g/l.

The data from the biometric measurements of the growth of the length of the root, hypocotyl, and sprout (cm) allow to objectively compare and evaluate the influence of the emulsifiers included in the study and applied concentrations (% v/v) on the initial growth (cm) and accumulation of fresh biomass (g) for one plant (Tables 5 and 6).

From the data analysis, it is evident that the addition of Polysorbate 80 to agar-gel has a statistically significant (P=0.05) indifferent effect on the growth of the seedlings of the target plants at concentrations in the range of 0.0125 to 0.8% v/v, while concerning the accumulation of fresh biomass, the neutral biological impact of the emulsifier is specified in a relatively narrower range - from 0.125 to 0.2% v/v, with insignificant differences being established between the target plants included in the study.

Species specificity was established in the reaction of the target plants included in the experiment with respect to the tested concentrations of the emulsifier Glyceryl Monostearate SE. Glyceryl Monostearate SE applied in concentrations above 0.025% v/v in *S. alba*, statistically significant (P=0.05) inhibits the growth of the root, hypocotyl, and seedling, while in *L. sativa*, statistically proven inhibition in the growth of the generative organs is established at the higher concentrations used - from 0.4 to 12.0% v/v. An inversely proportional relationship was established regarding the species sensitivity of the target plants included in the study after application of Olivem 1000. From the analysis of the experimental data presented in (Tables 5 and 6) it is evident that all applied concentrations (except the lowest of 0.0125% v/v) of the emulsifier Olivem 1000 reduced the growth of *L. sativa*, while in *S. alba*, statistically significant inhibition of the studied indicators was established at concentrations in the range from 0.1 to 12.0% v/v.

The mathematical-statistical analysis of variance, depending on the emulsifiers used and the applied concentrations on the growth of the generative organs (root, hypocotyl and seedling), shows that the relatively largest share of the total variation is occupied by Factor B (concentrations) (η^2 = 15.3%), followed by Factor A (emulsifiers) η^2 = 8.6%, while in terms of the formed fresh biomass (g) for one plant, the largest share of the total variation is occupied by Factor C (target plants) η^2 = 45.2%, followed by Factor B (η^2 = 15.4%) and a relatively weaker weight of Factor A η^2 = 8.8%. The values of the variances of the interaction of the studied factors determine a relatively low share of the total variation, η^2 is in the range from 0.8 to 4.5% 45.2%, although their influence is statistically proven at P=0.05. An exception is found only in the BxC relationship (η^2=17.8%) in terms of the accumulation of fresh biomass of the target plants included in the study, an indicator that is most strongly influenced by the biological impact of the emulsifiers included in the study. Regardless of the established differences in terms of the biological impact of the emulsifiers included in the study, the lower applied concentrations have an indifferent effect on the growth in length (cm) and the accumulation of fresh biomass of the seedlings in the target plants. They found significant differences in the biological response of the target plants to the applied concentrations of the emulsifiers, as follows: Polysorbate 80 (*S. alba* ≤0.4÷0.1% v/v and *L. sativa* ≤0.8÷0.2% v/v); Glyceryl Monostearate SE (*S. alba* ≤0.025÷0.0125% v/v and *L. sativa* ≤0.2% v/v) and Olivem 1000 (*S. alba* ≤0.05÷0.0125% v/v and *L. sativa* ≤0.0125% v/v) (Table 5 and 6).

Based on the experimental data obtained from the biometric measurements of the generative organs (root and hypocotyl) and analysis of the coefficient of allometry (CA) or proportionality in the increase in length (cm) and the accumulation of fresh biomass (g) of the root and hypocotyl, it is evident that the natural emulsifiers included in the study reduce the coefficient of proportionality with increasing concentration - CA from 3.9 to 80.7% in *S. alba* and from 3.1 to 71.8% in *L. sativa* in the increase in root and hypocotyl length, while in terms of the formation of fresh biomass, CA is respectively from 4.4 to 48.7% in *S. alba* and from 1.3 to 67.1% in *L. sativa* compared to the control (untreated) variants (Figures 1a-b, 2a-b and 3a-b).

Table 5. Effect of the included emulsifiers on the initial development (cm) of *Sinapis alba* L. and *Lactuca sativa* L.

Emulsifiers	**Concen-tration,** %v/v	**Target plants**					
		S. alba			***S. sativa***		
	Length, cm	Root	Hypocotyl	Seedling	Root	Hypocotyl	Seedling
Polysorbate 80	0.0	1.75de	5.33cde	7.08c	1.45c	2.45b	3.90c
	0.0125	1.50cde	5.63de	7.13c	1.45c	2.63b	4.08c
	0.025	2.33e	5.13cde	7.45c	1.50c	2.53b	4.03c
	0.05	1.60cde	5.83de	7.44c	1.33bc	2.50b	3.83c
	0.1	1.61de	5.47cde	7.07c	1.30bc	2.30b	3.60bc
	0.2	0.93abcd	6.16e	6.08bc	1.33bc	2.33b	3.67c
	0.4	1.10bcd	4.47cd	5.57bc	1.25bc	2.33b	3.58bc
	0.8	0.60abc	5.61de	6.21c	1.20bc	2.20b	3.40bc
	1.6	0.26ab	3.95c	4.22b	0.80b	1.83ab	2.63b
	3.0	0.00a	1.77b	1.77a	0.00a	0.52a	0.52a
	6.0	0.00a	0.70ab	0.70a	0.00a	0.00a	0.00a
	12.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Glycerol Monostearat **SE**	0.0	1.75b	5.33c	7.08d	1.45d	2.45c	3.90c
	0.0125	1.70b	5.36c	7.06d	1.43d	2.50c	3.93c
	0.025	1.23b	5.00c	6.23d	1.40d	2.58c	3.98c
	0.05	0.55a	3.17b	3.72c	1.30d	2.43c	3.73c
	0.1	0.18a	2.83b	2.92bc	1.14d	2.22c	3.36c
	0.2	0.00a	0.88a	0.88ab	1.20d	2.23c	3.43c
	0.4	0.00a	0.00a	0.00a	0.63c	1.50b	2.13b
	0.8	0.00a	0.00a	0.00a	0.30bc	1.42b	1.72b
	1.6	0.00a	0.00a	0.00a	0.24ab	1.44b	1.68b
	3.0	0.00a	0.00a	0.00a	0.00a	0.24a	0.24a
	6.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	12.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Olivem 1000	0.0	1.75c	5.33c	7.08c	1.45d	2.45d	3.90d
	0.0125	1.70bc	4.72c	6.42c	1.70d	1.98d	3.67d
	0.025	1.85c	5.05c	6.90c	0.78c	1.13c	1.90c
	0.05	1.10b	4.35c	5.45c	0.72c	0.88c	1.60c
	0.1	0.30a	1.83b	2.00b	0.60bc	0.74bc	1.34c
	0.2	0.00a	0.75ab	0.75ab	0.40b	0.25ab	0.65b
	0.4	0.00a	0.36a	0.36ab	0.00a	0.22ab	0.22ab
	0.8	0.00a	0.33a	0.04a	0.00a	0.22ab	0.22ab
	1.6	0.00a	0.23a	0.06a	0.00a	0.24ab	0.24ab
	3.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	6.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	12.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a

Independent action and interaction of the studied factors

Indicator		Root	Hypocotyl	Seedling	Factors	MS	η^2
Factor A (emulsifiers)	a_1	0.949b	3.040c	3.967c	A	8.63	4.7
	a_2	0.615a	1.708b	2.319b	B	15.34	46.3
	a_3	0.524a	1.307a	1.802a	C	0.39	0.1
Factor B (concentrations)	b_1	1.626g	4.006g	5.634f	AxB	0.85	5.1
	b_2	1.589g	3.879fg	5.469f	AxC	1.47	0.8
	b_3	1.528g	3.579fg	5.107ef	BxC	0.87	2.6
	b_4	1.097f	3.229ef	4.327e	AxBxC	0.29	1.7
	b_5	0.833ef	2.559de	3.359d	error	0.33	37.6
	b_6	0.661de	2.144cd	2.665cd	Intercept	224.82	
	b_7	0.511cd	1.515bc	2.020bc			
	b_8	0.327bc	1.583bc	1.857bc			
	b_9	0.166ab	1.183b	1.345b			
	b_{10}	0.00a	0.340a	0.340a			
	b_{11}	0.00a	0.117a	0.117a			
	b_{12}	0.00a	0.030a	0.030a			
Factor C (target plants)	C_1	0.661a	2.596a	3.217b			
	C_2	0.731a	1.441b	2.174a			

Legend: a, b, c, d, e, f, g, h, i – LSD statistically significant differences at P=0.05. Values marked with the same letter are statistically insignificant at P=0.05; MS–mean sum of squares-ANOVA; η^2-strength of influence of factors in factorial plane.

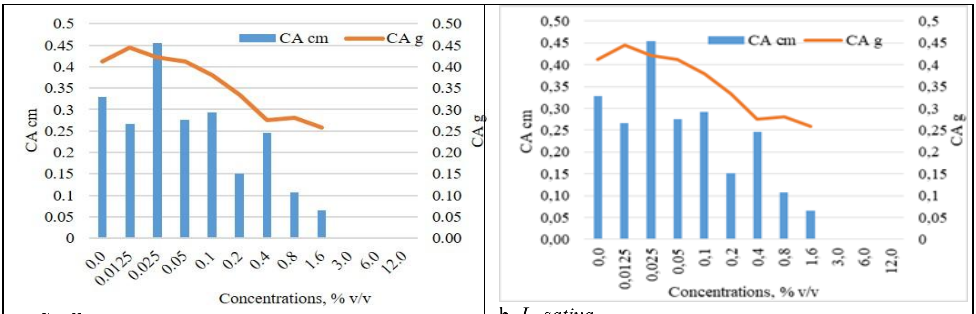
Table 6. Influence of the emulsifiers included in the study on the accumulation of fresh biomass (g) at *Sinapis alba* L. and *Lactuca sativa* L.

Emulsifiers	Concentrations, % v/v	Target plants					
		S. alba			***L. sativa***		
	Fresh biomass, g	Root	Hypocotyl	Seedling	Root	Hypocotyl	Seedling
Polysorbate 80	0.0	0.021e	0.051c	0.071gh	0.004d	0.021f	0.024f
	0.0125	0.024f	0.054f	0.078i	0.004d	0.021f	0.025f
	0.025	0.024f	0.057g	0.073h	0.003cd	0.019ef	0.021ef
	0.05	0.021e	0.051c	0.071gh	0.003cd	0.018def	0.020def
	0.1	0.019e	0.050c	0.069g	0.003cd	0.017def	0.020def
	0.2	0.017d	0.051c	0.056f	0.003cd	0.017def	0.020def
	0.4	0.014c	0.051c	0.045d	0.003cd	0.016d	0.019de
	0.8	0.014c	0.050c	0.049e	0.002bc	0.016d	0.018d
	1.6	0.008b	0.031d	0.043d	0.001ab	0.011c	0.012c
	3.0	0.000a	0.015c	0.015c	0.000a	0.008b	0.008b
	6.0	0.000a	0.008b	0.008b	0.000a	0.000a	0.000a
	12.0	0.000a	0.000a	0.000a	0.000a	0.000a	0.000a
Monostearat SE	0.0	0.021e	0.051f	0.071f	0.004d	0.021de	0.024d
	0.0125	0.020e	0.060g	0.080g	0.004d	0.023e	0.023d
	0.025	0.014d	0.044e	0.052e	0.004d	0.021de	0.023d
	0.05	0.008c	0.038d	0.048d	0.003cd	0.020d	0.023d
	0.1	0.005b	0.020c	0.025c	0.003cd	0.020d	0.023d
	0.2	0.000	0.007b	0.007b	0.003cd	0.020d	0.023d
	0.4	0.000	0.000a	0.000	0.002bc	0.016c	0.018c
	0.8	0.000	0.000a	0.000	0.001ab	0.016c	0.017c
	1.6	0.000	0.000a	0.000	0.001ab	0.016c	0.017c
	3.0	0.000	0.000a	0.000	0.000a	0.010b	0.010b
	6.0	0.000	0.000a	0.000	0.000a	0.000a	0.000a
	12.0	0.000	0.000a	0.000	0.000a	0.000a	0.000a
Olivem 1000	0.0	0.021d	0.051g	0.071d	0.004d	0.0213d	0.0243e
	0.0125	0.020d	0.051g	0.072d	0.001b	0.0057b	0.0110d
	0.025	0.021d	0.040e	0.061c	0.002c	0.0096c	0.0079c
	0.05	0.013c	0.047f	0.060c	0.001b	0.0051b	0.0061b
	0.1	0.005b	0.020d	0.025b	0.001b	0.0050b	0.0060b
	0.2	0.000a	0.007c	0.000a	0.001b	0.0003a	0.0010a
	0.4	0.000a	0.003b	0.000a	0.000a	0.0003a	0.0010a
	0.8	0.000a	0.002b	0.000a	0.000a	0.0003a	0.0010a
	1.6	0.000a	0.002b	0.000a	0.000a	0.0002a	0.0002a
	3.0	0.000a	0.000a	0.000a	0.000a	0.0000a	0.0000a
	6.0	0.000a	0.000a	0.000a	0.000a	0.0000a	0.0000a
	12.0	0.000a	0.000a	0.000a	0.000a	0.0000a	0.0000a

Independent action and interaction of factors

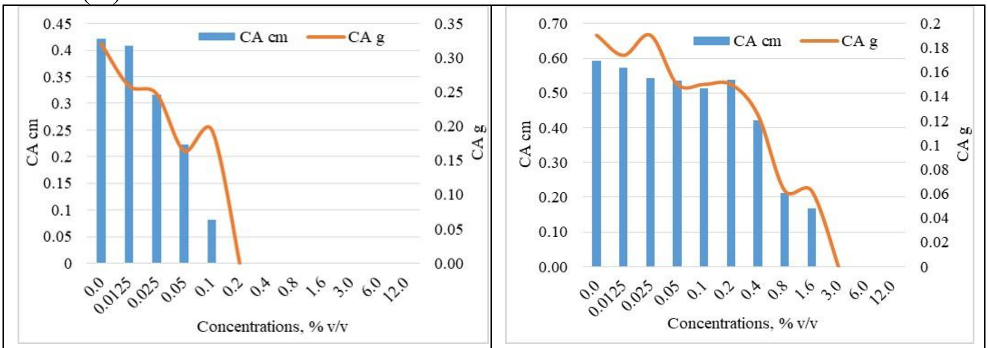
Indicator		Root	Hypocotyl	Seedling	Factors	MS	η^2
Factor A (emulsifiers)	a_1	0.0078b	0.0264c	0.0319	A	0.011585	8.8
	a_2	0.0038a	0.0168b	0.0201	B	0.040747	15.4
	a_3	0.0037a	0.0112a	0.0142	C	0.010871	45.2
Factor B (concentrations)	b_1	0.0123e	0.0360j	0.0478f	AxB	0.005989	4.5
	b_2	0.0121e	0.0346hi	0.0495f	AxC	0.000566	4.7
	b_3	0.0112e	0.0320hi	0.0372e	BxC	0.004280	17.8
	b_4	0.0082d	0.0307h	0.0366e	AxBxC	0.000379	3.2
	b_5	0.0061cd	0.0221g	0.0280d	error	0.000003	0.4
	b_6	0.0040bc	0.0171ef	0.0190c	Intercept	0.210483	
	b_7	0.0031b	0.0143de	0.0137bc			
	b_8	0.0028b	0.0140de	0.0141bc			
	b_9	0.0017ab	0.0101cd	0.0120b			
	b_{10}	0.0000a	0.0056bc	0.0056a			
	b_{11}	0.0000a	0.0014ab	0.0014a			
	b_{12}	0.0000a	0.0000a	0.0000a			
Factor C (target plants)	c_1	0.0086b	0.0253b	0.0318b			
	c_2	0.0017a	0.0110a	0.0124a			

Legend: a, b, c, d, e, f, g, h, i – LSD statistically significant differences at P=0.05. Values marked with the same letter are statistically insignificant at P=0.05; MS – mean sum of squares - ANOVA; η^2- strength of influence of factors in the factorial plane.



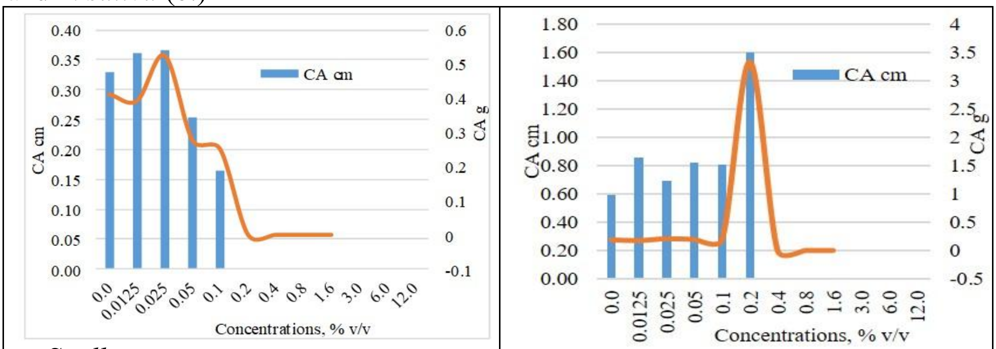
a. *S. alba* b. *L. sativa*

Figure 1. Coefficients of allometry (CA) proportionality in terms of growth (cm) and formed fresh biomass (g) of the root and hypocotyl, after application of Polysorbate 80 to the target plants included in the study at *S. alba* (a.) and *L. sativa* (b.)



a. *S. alba* b. *L. sativa*

Figure 2. Coefficients of allometry (CA) proportionality in terms of growth (cm) and formed fresh biomass (g) of the root and hypocotyl, after application of Glyceryl Monostearat SE to the target plants included in the study at *S. alba* (a.) and *L. sativa* (b.)



a. *S. alba* b. *L. sativa*

Figure 3. Coefficients of allometry (CA) proportionality in terms of growth (cm) and formed fresh biomass (g) of the root and hypocotyl, after application of Olivem 1000 to the target plants included in the study at *S. alba* (a.) and *L. sativa* (b.)

The biological influence of the emulsifiers included in the experiment, depending on the applied concentrations (from 0.0125 to 12.0% v/v), was assessed by comparing their regulatory effect on the growth (cm) and the accumulation of fresh biomass (g) of the seedling in the target plants *S. alba* and *L. sativa* by determining the dose-effect relationship, after interpolation of the data and approximation of the experimental results.

From the analysis of the results presented in Table 5, it is evident that the addition of Polysorbate 80 in the agar-gel causes a relatively weaker inhibitory effect on the growth (cm) (LC 50 = 1.5% v/v) and the accumulation of fresh biomass (g) of the (LC 50 = 1.2% v/v) in the two target plants *S. alba* and *L. sativa*, which determines their equivalent sensitivity to the emulsifier.

Based on the interpolation of the data to establish the dose-effect interaction when using the emulsifiers Glyceryl Monostearat SE and Olivem 1000, it can be summarized that the concentrations used have a strong depressing effect compared to the application of the emulsifier Polysorbate 80 on the growth (cm) (LC 50 from 0.04 to 0.6% v/v) and the accumulation of fresh biomass (g) of the seedling in both target plants - LC 50 varies in a relatively narrow range - from 0.01 to 0.07% v/v. An exception is found in *L. sativa*, after adding Olivem 1000 to the agar-gel, which has a relatively weaker biological impact on the accumulation of fresh biomass (g) (LC 50 = 1.4% v/v) of the seedling in the target plant.Regardless of the proven species sensitivity of the target plants included in the study to the biological impact of the applied emulsifiers, specificity in their phytotoxic effect was established with respect to the initial development of their generative organs (root and hypocotyl). The LC 50 values in terms of growth (cm) and accumulation of fresh biomass (g) of the seedling at the two target plants, depending on the biological impact of the emulsifiers included in the study, allow to determine and compare the sensitivity.

From the analysis of the data it is evident that the emulsifiers included in the study have a stronger inhibitory effect on the growth and accumulation of fresh biomass of the root biomass compared to the hypocotyl, with the LC 50 values determined in terms of the formed root biomass being from 0.6 to 6.8 times higher compared to the growth and accumulation of fresh biomass of the hypocotyl in the target plants (Table 7).

For a more complete assessment of the biological influence of the emulsifiers included in the study on the initial development of the target plants, a ranking (Re) was performed based on the determined LC 50 values in relation to LC 50 (laboratory seed germination), LC 50 (root growth, cm or fresh biomass accumulation, g) and LC 50 (hypocotyl growth, cm or fresh biomass accumulation, g), according to Bashar *et al.* (2023). From the ranking (Re) performed, it is evident that the application of Polysorbate 80 has the weakest influence on the initial development of the target plants included in the experiment (Re is in the range from 4.151 to 3.452), which determines a high tolerance of *S. alba* and *L. sativa* to the emulsifier used. Significant differences are found when using Glyceryl Monostearate SE. The ranking shows that *L.*

sativa has a significantly higher rank (Re is in the range from 1.931 to 3.679) compared to *S. alba* (Re – 0.171 to 0.174), which determines the high toxicity of the emulsifier Glyceryl Monostearat SE to *S. alba* and its appropriateness for use in screening laboratory studies.

Table 7. LC 50 of the emulsifiers included in the study on the growth of generative organs (root, hypocotyl and seedlings) in the target plants

LC_{50}			Emulsifiers					
			Polysorbate 80		Glyceryl Monostearat SE		Olivem 1000	
Target plants			*S. alba*	*L. sativa*	*S. alba*	*L. sativa*	*S. alba*	*L. sativa*
Growth, cm	Root	LC_{50}	0.363	0.612	0.037	0.340	0.076	0.050
		LC_{50} LL	0.292	0.499	0.033	0.292	0.065	0.039
		LC_{50} UL	0.452	0.751	0.042	0.395	0.089	0.063
	Hypocotyl	LC_{50}	2.454	1.631	0.080	0.953	0.089	0.031
		LC_{50} LL	2.106	1.381	0.071	0.799	0.078	0.024
		LC_{50} UL	2.861	1.925	0.090	1.137	0.102	0.041
	Seedling	LC_{50}	1.498	1.519	0.140	0.648	0.079	0.045
		LC_{50} LL	1.261	1.295	0.125	0.548	0.070	0.037
		LC_{50} UL	1.779	1.781	0.156	0.767	0.089	0.054
Fresh biomass formed, g	Root	LC_{50}	1.570	0.737	0.040	0.399	0.061	0.008
		LC_{50} LL	0.930	0.444	0.035	0.294	0.054	0.004
		LC_{50} UL	2.650	1.224	0.045	0.542	0.068	0.017
	Hypocotyl	LC_{50}	1.582	1.350	0.074	2.642	0.089	0.009
		LC_{50} LL	1.463	0.996	0.065	1.931	0.077	0.006
		LC_{50} UL	1.711	1.830	0.083	3.617	0.103	0.013
	Seedling	LC_{50}	1.224	1.223	0.061	1.349	0.070	0.011
		LC_{50} LL	0.983	0.914	0.053	1.061	0.063	0.007
		LC_{50} UL	1.524	1.637	0.070	1.715	0.078	0.016

Note: LL - lower Limit LC 50, UL – Upper Limit LC 50

The results obtained with Olivem 1000 are similar. The emulsifier causes a strong inhibitory effect on the initial development of the target plants, which determines their inability to be used in screening laboratory studies using *S. alba* and *L. sativa* as recipient target plants.

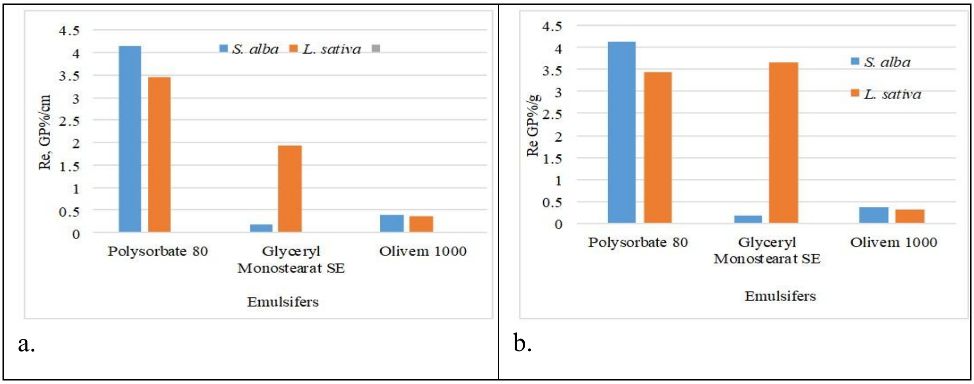


Figure 4. Rank (Re) in relation to the relationship between LC 50 of laboratory germination seeds and LC 50 depending on the growth of the root and hypocotyl, cm (a.); Rank (Re) in relation to the relationship between LC 50 of laboratory germination seeds and LC 50 depending on the formed fresh biomass of the root and hypocotyl, g (b.)

Figures 4a-b. Rank (Re) of the biological influence of the emulsifiers included in the study on the initial development of the target plants included in the experiment.

Evaluating the integral and complex biological impact of the emulsifiers included in the study, regardless of the type of target plants, allows them to be ranked (Re total) in the following descending order: with the weakest total biological impact Re total = 15.385 was found when using Polysorbate 80, followed by Glyceryl Monostearate SE (Re total = 5.955) and with the strongest biological impact (Re total = 1.421) when using Olivem 1000 (Figure 5).

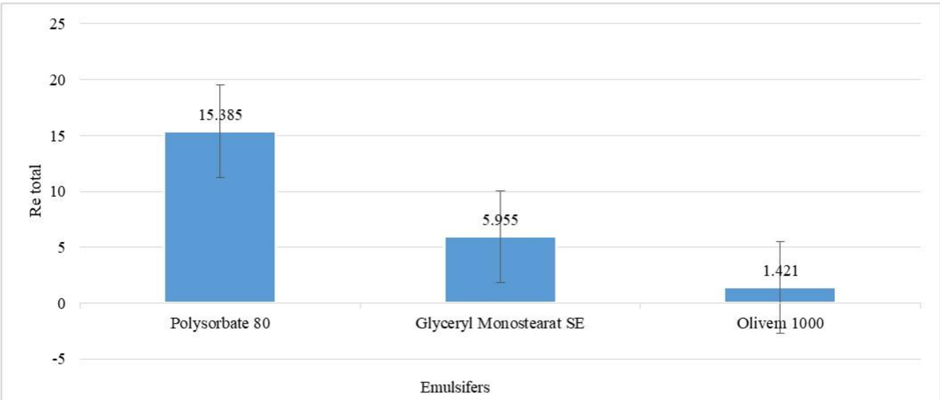


Figure 5. Ranking (Re total) of the biological influence of the emulsifiers included in the study

By assessing the integral influence of the emulsifiers included in the study, through the generalized growth index (GI) of the target plants depending on the average values of laboratory seed germination (SG%) and seedlings length (root + hypocotyl) (SL), their biological impact can be differentiated according to the applied concentrations (Figures 6 and 7).

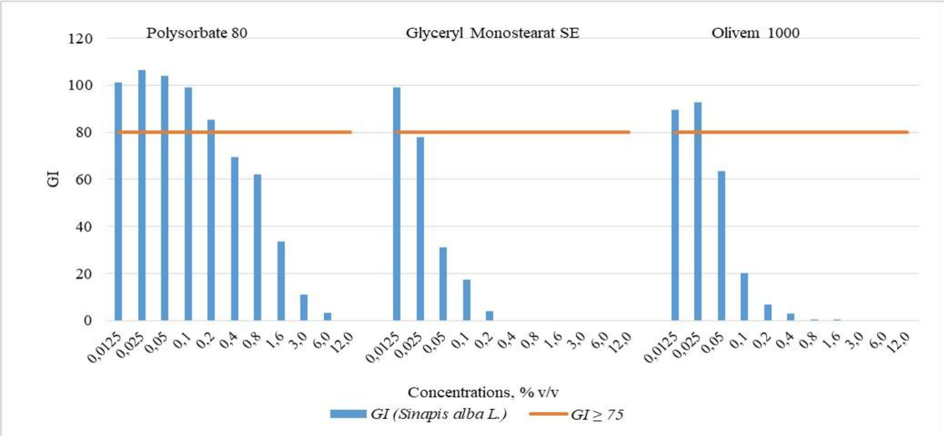


Figure 6. Integral effect of the emulsifiers included in the study on the generalized development index (GI) of *Sinapis alba* L. under laboratory conditions

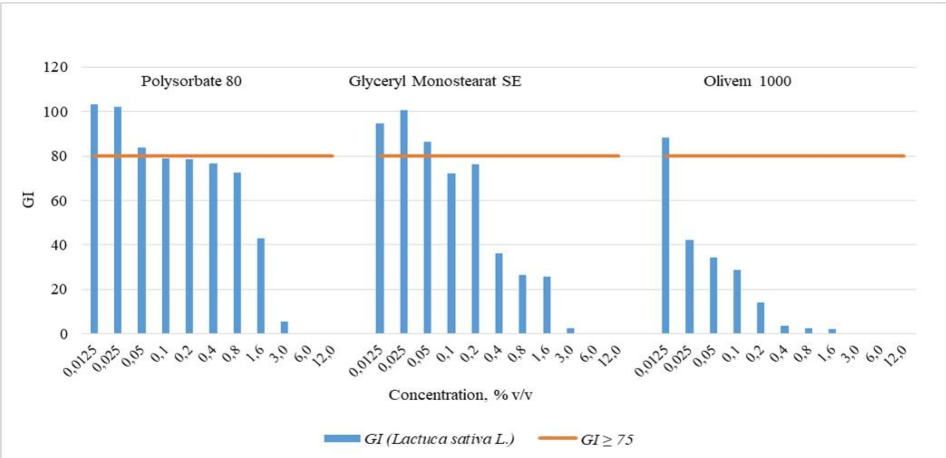


Figure 7. Integral effect of the emulsifiers included in the study on the generalized growth index (GI) of *Lactuca sativa* L. under laboratory conditions

From the analysis of the results presented in Figures 6 and 7, it is evident that the emulsifiers included in the study have a biological effect (from a stimulating to an inhibitory effect) on the target plants depending on the applied concentrations. The lowest applied concentrations (0.0125 and 0.025% v/v) of Polysorbate 80 cause a stimulating effect on the initial development of the target plants included in the study (GI ≥100%). With increasing concentration from 0.05 to 0.2% v/v, they have an indifferent biological effect on both test plants (GI ≥ 79%), while concentrations in the range from 1.6 to 3.0 % v/v cause a strong inhibitory effect (GI ≤ 43%) or a lethal effect at the highest applied concentrations. Significant differences in the biological impact on the target plants were found when using the emulsifiers Glyceryl Monostearate SE and Olivem 1000 compared to Polysorbate 80, and significant differences in the biological response of the test plants were found. The application of Glyceryl Monostearate SE had a stronger depressing effect on the generalized growth index (GI) in *S. alba* compared to *L. sativa.* From the analysis of the data presented in Figure 6 and 7 it is evident that an indifferent influence of the emulsifier was found in a very narrow range at the lowest concentrations studied, respectively in *S. alba* – from 0.0125 to 0.025% v/v, at GI ≥ 78.1%, while in L. sativa it was in the range depending on the applied concentrations from 0.0125 to 0.2% v/v (GI ≥ 76.2%), disproportionately with increasing the concentration of the emulsifier in the agar-gel. With increasing concentration of Glyceryl Monostearate SE, a strong inhibitory effect was found in the range of 0.5 – 0.2% v/v in *S. alba* and from 0.4 to 3.0% v/v in *L. sativa*. Concentrations above 0.4% v/v caused a lethal effect in *S. alba*, while in *L. sativa*, death of the test plants was found after application of concentrations above 3.0% v/v.

The emulsifier Olivem 1000 has the highest inhibitory effect on initial development, which at the lowest applied concentrations (0.0125 and 0.025% v/v) has an indifferent effect on the initial development of the target plants (GI ≥ 88.4%). With increasing concentrations above the specified range, a strong inhibitory effect is established, and above 0.3% v/v - a lethal effect on both target plants.

CONCLUSIONS

Following the experimental approach, a test was developed to optimize the application of emulsifiers when performing screening allelopathic studies in laboratory conditions:

- ➢The biological influence of the emulsifiers used (Polysorbate 80, Glyceryl Monostearate SE, and Olivem 1000) on the laboratory germination of seeds and the initial growth of *S. alba* and *L. sativa*, used as target plants, was proven.
- ➢It was found that Polysorbate 80 can be applied in concentrations ranging from 0.05 to 0.2% v/v, while Glyceryl Monostearate SE and Olivem 1000 can be used in concentrations not higher than 0.025% v/v, as emulsifiers when performing screening laboratory studies to establish the biocidal effect of essential oils.
- ➢Further research is needed, including validation of the experimental results obtained in laboratory and in-vessel trials, to establish the influence of emulsifiers when combined with essential oils or hydrolates from plants with proven allelopathic potential.

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