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Optimizing allelopathy screening bioassays by using Tagetes sp. - water absorption capacity of the seeds

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Abstract. The aim of the present study is to: i) analyze the water absorption capacity of seeds of some *Tagetes* sp.; ii) compare the water absorption of *Tagetes* sp. and *Lactuca sativa* L. seeds in order to assess the potential of genus *Tagetes* as test object for laboratory screening studies for establishing allelopathic interference in plant communities. *Tagetes erecta* (local population from Germany), *Tagetes patula* (local population from Germany), and *Tagetes patula* variety "Usmivka" (Bulgarian variety) have been included in the study. Equivalence in the water absorption capacity (Ws%) of the seeds of studied *Tagetes* sp. and *Lactuca sativa* L. variety "Great Lakes" was found, with the maximum of water absorption occurring at the first hour and stopping by the fourth hour, regardless of the temperature range in which the imbibition takes place. The significant differences in the cumulative values of the average water absorption capacity of the *Tagetes* sp. seeds compared to *Lactuca sativa* L. variety "Great Lakes" (Ws% average ranged from +5.68 to +83.27%), indicate that the species of genus *Tagetes* can be successfully used in laboratory screening studies to establish allelopathic interference under in vitro conditions. Further research is needed to establish seed water absorption capacity (Ws%) of *Tagetes* sp. depending on the type of aqueous extracts used in allelopathic studies (cold or temperate), as well as of the concentrations applied.

Key words: water imbibition, soaking, seeds, *Tagetes*.

Introduction

Allelopathy is widely studied from more than 50 years but still there are some challenges in exploration of plant interactions both in the field and in the lab. Many authors often revealed at contradictory results, confirming the inhibitory or stimulatory effect of various plants on seed germination of other plant species (Kadioglu et al., 2005; Marinov-Serafimov et al., 2015; Treber et al., 2015). Difficulties in such studies are found to be due to the lack of unifying methods for performing laboratory and field experiments, including growth medium, extract preparation, microbial contamination, test plant selection, etc.

Seed germination is a critical phase in the life cycle of most plant species, especially the cultural ones (Ernst, 1998). This process begins with the uptake of water by the dry seed (imbibition) and its swelling and ends with the germination of the radicle through all the seed coats (Bewley, 1997).

Studies on the water absorption capacity of seeds in relation to their use in screening laboratory studies to establish allelopathic interference between plant species are sporadic. According to studies by many authors (Turk & Tawaha, 2003; Chong et al., 2002; Teerarak et al., 2012; Lin et al. 2019; El-Maarouf-Bouteau, 2022) the seeds of used test plants in allelopathic studies require a good supply of water to activate the metabolic processes in the seeds related to their water absorption, respectively and the available allelochemicals in the aqueous extracts. of seeds can be limited by multiple factors The water absorption capacity of seeds is determined by many factors: type and size of seeds, wettability, water permeability of the seed coat, oxygen absorption and others (de Pinho et al., 2004). According to the summary studies of Bhardwaj et al. (2014), Ali & Elozeiri (2017), Makena et al. (2018), Savaedi et al. (2019) and Sghaier et al. (2022), the process of seed water absorption depends on many factors related to the chemical composition of the seeds (content of protein, carbohydrates, lipids, etc.), the temperature at which it takes place, the size of the seeds (fractions), the stage of maturity, vitality, as well as and from their duration of storage. According to the studies of Sapirstein et al. (2018) and Wong et al. (2019), the water absorption capacity related to blocking of specific enzymes, hydrolysis of proteins, fats, seed coat thickness, etc.

A number of authors recommend the use of the test plant *Lactuca sativa* L. to determine the allelopathic potential of plant species in the laboratory (Macias et al., 2000; Yasmin et al., 2011; Mahmoodzadeh et al., 2015; Carvalhoa et al., 2019; Wang et al., 2020). The species is defined as an ideal screening agent due to its high rate of germination and growth, as well as its high sensitivity to various allelochemicals and pesticides (Rashid et al., 2010; Al Harun et al., 2014; Sun et al., 2022; França de Ferreira et al., 2023).

Available scientific literature reports data on the allelopathic potential and possibilities of *Tagetes* species to be applied in organic agriculture (Zunino et al., 2005; López et al., 2008; Santos et al., 2015; Wongsnansilp et al., 2022). They are well known to exert various bactericidal, nematicidal, fungicidal and insecticidal action (Hooks et al., 2010; Piña-Vázquez et al., 2017; Santos, 2022) of their above-ground biomass, but there is a lack of information on the water absorption capacity of the seeds and their potential as test plants in allelopathic screening studies in vitro.

In this aspect, the aim of the present study is to: *i*) analyze the water absorption capacity of seeds of some *Tagetes sp.; ii*) compare the water absorption of *Tagetes sp*. and *Lactuca sativa* L. seeds in order to assess the potential of genus *Tagetes* as test object for laboratory screening studies for establishing allelopathic interference in plant communities.

Materials and Methods

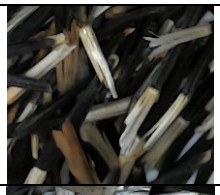
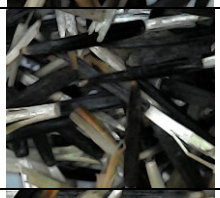
The study was conducted in 2023-2024 under laboratory conditions at the Institute of Decorative and Medicinal Plants - Sofia. The samples of the *Tagetes* genus from the working collection of the Institute of Decorative and Medicinal Plants - Sofia (Zapryanova, 2014) have been use as presented in Table 1. Seeds of the test plant *Lactuca sativa* L., variety "Great Lakes", were used for comparison as a standard.

Seeds of each one of the studied species were manually calibrated by selecting 100 seeds per species, weighing (g), and then placing into Petri dishes with a diameter of 40 mm between Filtrak 383 filter paper. 5 ml of pre-tempered distilled water at 15, 20 and 30°C ± 2°C were sealed with Parafilm "M" paraffin tape, then placed in the dark in thermostats at 15, 20 and 30°C ± 2°C. Each variant was set in four replicates. After the expiration of each absorption time (t_{abs}) – 1, 2, 3, 4, 5, 6 and 7 h, the available water in the Petri dishes was decanted, and the seeds were separated, using a Buchner funnel connected to a vacuum pump. Immediately before weighing them on an analytical balance (0.000g), they were surface-dried on three-layer absorbent paper without pressing. The formula of Hidayati et al. (2001) was used to determine the water absorption capacity (%Ws) (g absorbed H_2O/g seeds). (2001):

$$\boldsymbol{W_s\% = [(W_i - W_d)/W_d]/\times 100}$$

where: W_i – mass of seeds (g) after each absorption time (t_{abs}) – 1, 2, 3, 4, 5, 6 and 7 h, W_d – mass of air-dry seeds (g) and/or for each previous absorption time (t_{abs}), until maintaining a constant weight of the samples.

Table. 1. List of the specimens used in the present study

№	Scientific name	Origin	Genotype	Nomenclature	
1	*Tagetes erecta* L.	Germany	Local population	TE – 1	
2	*Tagetes patula* L.	Germany	Local population	TP – 1	
3	*Tagetes patula* L.	Bulgaria	Variety "Usmivka"	TP – 2	
4	*Lactuca sativa* L.	Germany	Variety „Great Lakes"	LS	

Seed water absorption rate (R – g absorbed H_2O/h) was determined for each absorption time (t_{abs}) according to the formula of Mamonov and Kim (1978):

$$\boldsymbol{R = \frac{(W_2 - W_1)}{t_2 - t_1}}$$

where: W_1 mass of air-dry seeds, g at the initial time of reading; W_2 seed mass, g for each tracked time (tabs); (t_2 - t_1) – duration of seed soaking in distilled water, h.

Mathematical-statistical processing of the experimental results was performed with the program product STATGRAPHICS Plus for Windows Version 2.1. by the ANOVA method, one-way and two-way analysis of variance using Fisher's LSD test at a confidence interval of 95% and error α = 0.05. The strength of factor influence was determined by η^2 under reliable factor variance (Plochinsky, 1967; Lidansky, 1988). Hierarchical cluster analysis was used to identify the similarity and similarity of the water absorption capacity (Ws%) of the samples included in the study. The Euclidean distance between them was used as a measure of dissimilarity (Hair et al., 1987).

Results and Discussion

Unfavorable conditions of the environment have a strong impact on the germination processes (Vince & Zoltan, 2011), that is why the seed germination is a critical phase of plant development

The water absorption capacity of the seeds (Ws%) of the *Tagetes* specimens included in the study (Table 1) varies in a wide range (Ws% average from 9.32 to 20.54%), according to their biological characteristics and depending on the applied temperatures (from 15 to 30°C). It is relatively weaker at Ws% average at 15°C (13.94%), while when imbibing the seeds at 20 and 30°C it increased from 1.15% (Ws% average at 20°C = 14.10%) to 28.13% (Ws% average at 30°C = 17.81%). The observed differences are statistically significant ($P<0.05$), compared only to the highest imbibition temperature used – 30°C (Fig. 1).

From the regression analysis performed in relation to the water absorption capacity of the seeds (Ws%), a linear dependence Y = 13.625 + 0.505 x, r = 0.586 was established; R^2 = 34.3% with uncertainty coefficient K^2=65.66% of the *Tagetes* sp. Double-reciprocal or Lineweaver-Burke dependence on the applied imbibition temperatures

of the type Y = 1/(0.0249+0.364/x), r = 0.574; R^2 = 33.0%; K^2=67.05%, have been statistically proven at P < 0.01%.

The maximum water absorption of the seeds of *Tagetes* sp. occurs in the first hour, and in the fourth hour it ceases, regardless of the temperature at which the imbibition is carried out (Fig. 2, 3 and 4). Regardless of the equivalence in the established maximum seed water absorption (Ws%) of *Tagetes sp.* included in the study, a species-specific response was established (Fig. 5). The highest water absorption capacity was reported for TP – 2 (Ws% average = 19.58%), followed by TE – 1 (Ws% average = 14.83%) and relatively weakest for TP – 1 (Ws% average = 11.41%), the differences being statistically significant at P<0.05.

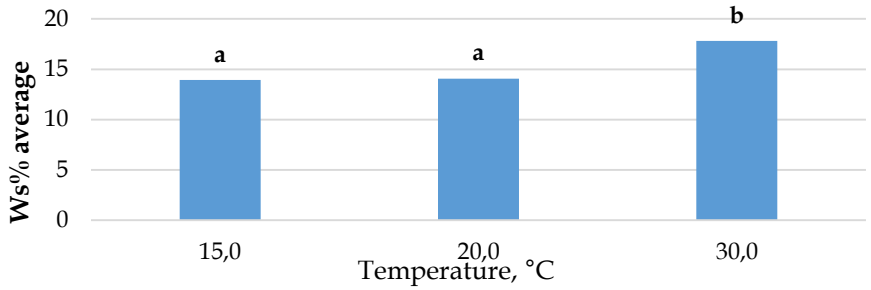


Fig. 1. Effect of temperature range on the water absorption capacity of *Tagetes* seeds
Legend: LSD multiple range test, a, b. Treatments bearing the same letter are not significantly different at P<0.05.

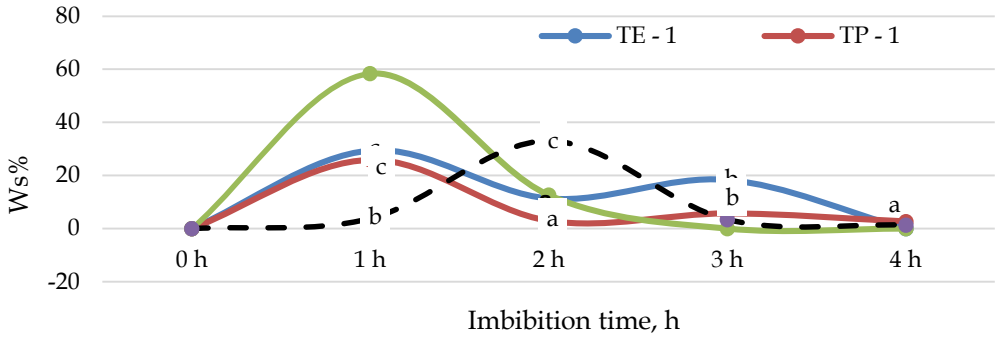


Fig. 2. Water absorption capacity of seeds of *Tagetes* sp. and *Lactuca sativa* at 15°C
Legend: TE – 1 - Tagetes erecta L.; TP – 1 and TP – 2 Tagetes patula L.; LS - Lactuca sativa L. a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

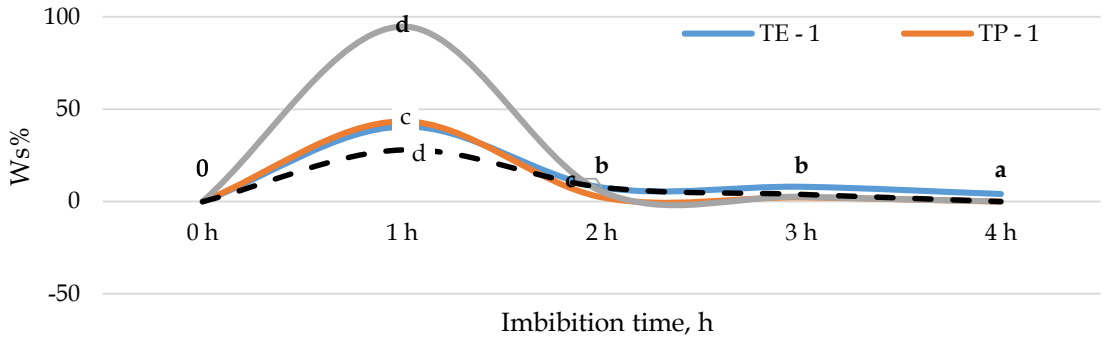


Fig. 3. Water absorption capacity of seeds of *Tagetes* sp. and *Lactuca sativa* at 20°C
Legend: TE – 1 - Tagetes erecta L.; TP – 1 and TP – 2 Tagetes patula L.; LS - Lactuca sativa L. a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

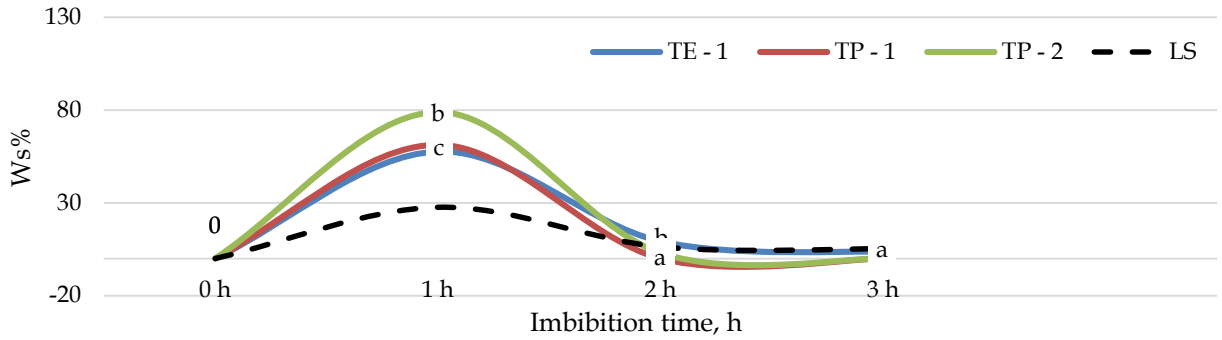


Fig. 4. Water absorption capacity of seeds of *Tagetes* sp. and *Lactuca sativa* at 30°C
Legend: TE – 1 - Tagetes erecta L.; TP – 1 and TP – 2 Tagetes patula L.; LS - Lactuca sativa L. a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

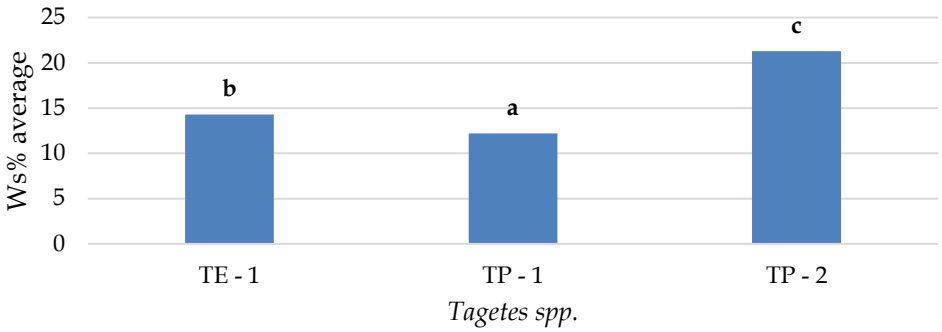


Fig. 5. Average water absorption capacity of seeds of studied *Tagetes* sp.
Legend: TE – 1 - Tagetes erecta L.; TP – 1 and TP – 2 Tagetes patula L.; a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

The results of the two-factor variance analysis performed to establish the weight of the factors (η^2) in the hierarchical distribution of variation on the water absorption capacity of the seeds of the *Tagetes* species included in the study (Factor A) and the influence of the applied temperature range (Factor B) show that Factor A (η^2 = 69.93) takes relatively the largest share of the total variation, followed by Factor B (η^2 = 19.98%). The values of the variances of the interaction of the studied factors A × B determine relatively the smallest share of the total variation (η^2 = 10.11), although their influence is statistically significant at P<0.05.

Table 2. Analysis of variance and degree of influence of the factors on the water absorption capacity of the *Tagetes* species included in the study

Influence of factors	Water absorption capacity, Ws%	
Factor A *Tagetes* spp.	14.29^b 12.21^a 21.30^c	
Factor B *Temperature, °C*	14.83^b 11.41^a 19.59^c	
Factors	*MS*	η^2
A	202.25	69.93
B	57.80	19.98
A × B	14.62	10.11

Analogous are the obtained experimental results when tracking the water absorption capacity of the seeds (WS%) of *Lactuca sativa* L. (LS), accepted as a standard. From the analysis of the data presented in Fig. 6, it is evident that the water absorption capacity (Ws%) of LS follows the established relationships in *Tagetes* species. The maximum water absorption of LS seeds occurs in the first hour at the applied higher temperature range – 20 and 30°C, while at 15°C – in the second hour of imbibition. Irrespective of the applied temperatures, LS seed water absorption ceased by the fourth hour.

Changes in the average water absorption capacity (Ws% average) of LS seeds depended on the temperature range used. With an increase in temperature above 20°C, a statistically proven increase ($P<0.05$) in water absorption is found, which exceeds the reported indicator to 63.25% (Fig. 7).

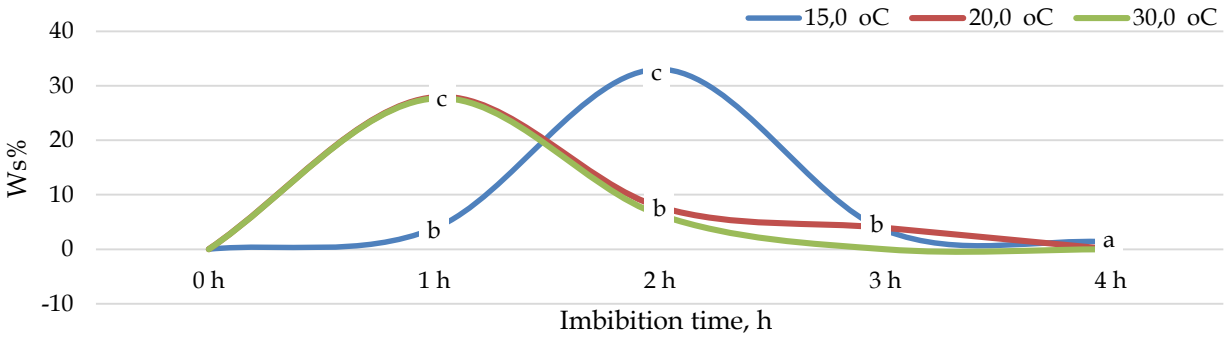


Fig. 6. Water absorption capacity of seeds of *Lactuca sativa* L. (LS) depending on imbibition temperature

Legend: a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

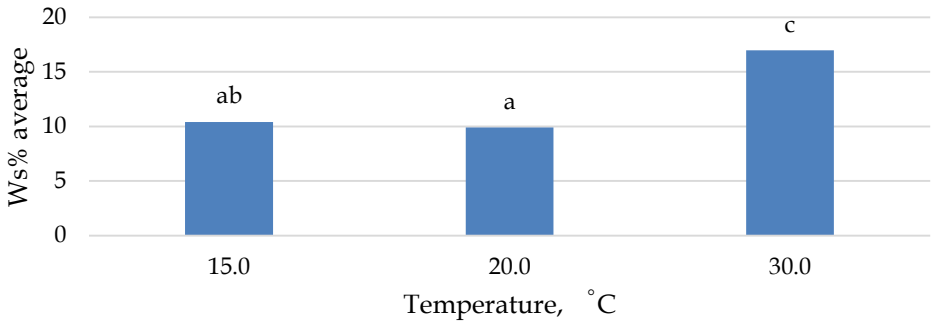


Fig. 7. Average water absorption capacity of seeds of *Lactuca sativa* L. (LS)

From the analysis of the data presented in Table 3, it is clear that as the duration of the absorption time (t_{abs}) increases, the water absorption capacity of the seeds of the *Tagetes* species, as well as of *Lactuca sativa* L., decreases disproportionately, after the first hour of imbibition, but the total amount of absorbed water increases.

The results obtained when tracking the rate of water absorption (R– g/H_2O/h) are also similar (Table 3). The rate (R – g/H_2O/h) of water absorption of studied seeds is the highest in the physico-chemical phase of imbibition with t_{abs} for up to 1 h, then it decreases and at 3-4 h it ceases, not being detected statistically significant differences ($P<0.05$) depending on the applied temperature range from 15 to 30°C. Relatively higher is the rate of water absorption (R- g/H_2O/h) of the seeds at a temperature of 20 or 30°C compared to that in the lowest temperature used – 15°C. An exception to the described dependence was found in sample TP - 2, where the rate of water absorption (R – g/H_2O/h) of the seeds stopped already in the second hour after soaking them in distilled water at the lowest used imbibition temperature (15°C).

Table 3. Water absorption dynamics of seeds of studied *Tagetes* sp. and *Lactuca sativa* L. Variety Great Lakes (used as a standard) depending on imbibition time, h

Variants	t°C	Characteristics	Imbibition time, h				
			1	2	3	4	5
TE - 1	15	Wa%	129.4	144.1	170.6	170.6	
		R	0.100	0.050	0.090	0.0	
TP - 1		%	125.9	129.6	137.0	137.0	
		R	0.070	0.010	0.020	0.0	
TP - 2		%	158.3	178.3	178.3		
		R	0.140	0.050	0.050		
LS		%	103.8	138.1	142.9	144.8	144.8
		R	0.004	0.036	0.005	0.002	0.0
TE - 1	20	%	140.5	151.4	163.5	170.3	170.3
		R	0.120	0.032	0.036	0.020	0.0
TP - 1		%	143.3	146.7	150.0	150.0	
		R	0.130	0.010	0.010	0.0	
TP - 2		%	194.7	205.3	210.5	210.5	
		R	0.180	0.020	0.010	0.0	
LS		%	127.9	137.8	143.2	143.2	
		R	0.031	0.011	0.006	0	
TE - 1	30	%	157.7	172.3	178.5	178.5	
		R	0.150	0.038	0.016	0.0	
TP - 1		%	161.3	161.3			
		R	0.190	0.0			
TP - 2		%	179.0	184.2	184.2		
		R	0.150	0.010	0.0		
LS		%	127.7	135.7	142.9	142.9	
		R	0.031	0.009	0.008	0.0	
Average							
Tagetes spp.		%	163.7	152.5	113.0	18.9	
		R	0.024	0.026	0.002	0.0	
Lactuca sativa		%	137.2	143.0	143.6	48.3	
		R	0.019	0.006	0.001	0.0	

Legend: Wa% - percentage of absorbed water depending on the mass of dry seeds, h; R - Seed water uptake rate (R – g absorbed H_2O/h); TE – 1 - Tagetes erecta L.; TP – 1 and TP – 2 Tagetes patula L.; LS - Lactuca sativa L.; a, b, c - LSD multiple range test. Treatments bearing the same letter are not significantly different at P<0.05.

The obtained experimental results are in agreement with that reported by Costa et al. (2018, 2022) according to which, the water absorption capacity (Ws%) and water absorption rate (R– g/H_2O/h) of seeds are highest in the initial hours (1.5 and 3 h) of their exposure to water and decreases with increasing soaking period. Although water absorption capacity (Ws%) and seed water absorption rate (R– g/H_2O/h) are determined by many factors (chemical composition, structural features of the seed's cover, water permeability, etc.) (Miano et al., 2015; Costa et al., 2022), no significant differences were found in the average values of these indicators when comparing the samples of the *Tagetes* genus, included in the study to *Lactuca sativa* L., accepted as a standard. In order to identify the similarity of the water absorption capacity (Ws%) of the between them, a hierarchical cluster analysis was used for grouping based on similarity and remoteness. Euclidean distance was used as a measure of distance, and the results of the analysis were presented graphically through dendrograms (Fig. 8 and 9).

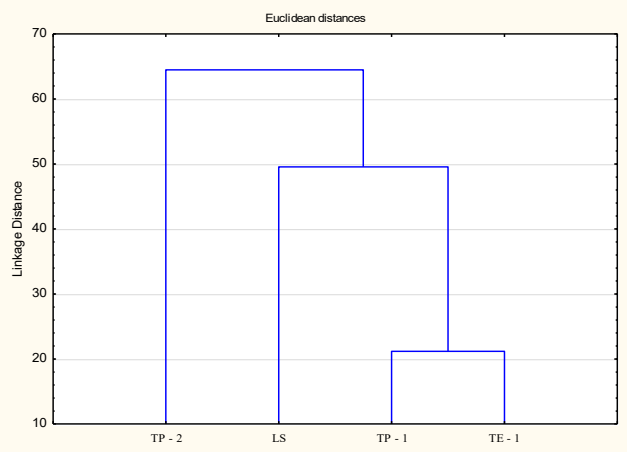


Fig. 8. Dendrogram based on the average intergroup distances depending on the water absorption capacity (Ws%) of *Tagetes sp.* and *L. sativa* seeds

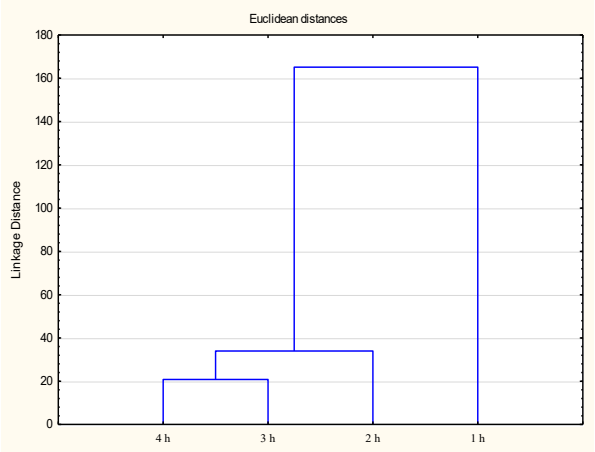


Fig. 9. Dendrogram based on the average intergroup distances depending on the intensity of water absorption (h) of *Tagetes sp.* and *L. sativa* seeds

From the cluster analysis, it is clear that TP - 2 stands out with the relatively highest water absorption capacity (Ws%) in comparison with *Lactuca sativa* L. Variety Great Lakes, accepted as a standard, which determines the distance (the largest Euclidean distance) with respect to indicator studied, according to the rescaled distance of the dendrogram at which the cluster was formed. With a relatively lower water absorption capacity (Ws%) compared to *Lactuca sativa* L. Variety Great Lakes, and united by similarity in a main cluster, are the samples TP - 1 and TE - 1 from the *Tagetes* genus included in the study (Fig. 8).

Three clusters are formed when comparing the hydration of the studied seeds depending on their imbibition duration (h) (Fig. 9). Relatively low water absorption capacity (Ws%) values from 3 and 4 h are found to be closest (smallest Euclidean distance), while the most distant cluster (largest Euclidean distance) includes the values at 1-st h, determining the highest hydration of the seeds after soaking them in water, regardless of the temperature range. A relatively intense kinetics in the water absorption of the seeds was recorded in the 2-nd hour of their imbibition – it is lower than in the first hour of their soaking, but with a relatively higher water absorption capacity compared to 3 and 4 hours, which causes its separation into an independent cluster.

The obtained experimental results are consistent with Baskin (1998), Foschi et al. (2020) and Pompelli et al. (2023), according to which seed water absorption is a three-phase process. Phase I - rapid water uptake, followed by phase II, where the seed water absorption is relatively slower reaching a plateau. Phase III is known as the water absorption phase after seed germination, which only viable seeds can reach.

Therefore, the statistically insignificant differences, found in the intensity of water absorption of the seeds of the studied *Tagetes* species in the first hour after soaking in water in comparison with the standard *Lactuca sativa* L., can be regarded as a prerequisite for their potential as test plants in bioassays. Data revealed that they can be successfully used as recipient test plants when performing laboratory screening studies to establish allelopathic interference in plant communities, since lettuce is one of the most commonly used species for this goal (Fujii et al., 1990, 2003; Valcheva et al., 2017; Meriem et al., 2019; Akazawa & Kato-Noguchi, 2023).

The established equivalent intensive water absorption up to the first hour of soaking the seeds of the genus *Tagetes*, as well as of *Lactuca sativa* L., is a prerequisite for a possible faster entry of the available allelochemicals (Sangeetha & Baskar, 2015), as the same are extremely unstable and short-lived, having a complex chemical structure, (Macias et al., 2003; Yoneyama & Natsume, 2010). During the extraction of allelochemicals from the plant material, it is also not taken into account that their content in aqueous solutions can increase

due to the diffusion of hydrophilic compounds compared to lipophilic ones, as well as it is possible to isomerize those that probably are not available in agrocenoses under field conditions (Francisco et al., 2004; Cheng & Cheng, 2015).

Conclusions

Equivalence in the water absorption capacity (Ws%) of the seeds of studied *Tagetes* sp. and *Lactuca sativa* L. variety "Great Lakes" was found, with the maximum of water absorption occurring at the first hour and stopping by the fourth hour, regardless of the temperature range in which the imbibition takes place.

The significant differences in the cumulative values of the average water absorption capacity of the *Tagetes* sp. seeds compared to *Lactuca sativa* L. variety "Great Lakes" (Ws% average ranged from +5.68 to +83.27%), indicate that the species of genus *Tagetes* can be successfully used in laboratory screening studies to establish allelopathic interference under in vitro conditions

Further research is needed to establish seed water absorption capacity (Ws%) of *Tagetes* sp. depending on the type of aqueous extracts used in allelopathic studies (cold or temperate), as well as of the concentrations applied.

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