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Suitability of colloidal Nano Gold for application in performing allelopathic screening studies in laboratory conditions

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Abstract

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Nanomaterials are widely used in medicine and industry, while in agriculture their application is relatively limited, but they have the potential to be included in screening studies to establish allelopathic interference in plant communities. Therefore, a key element is to establish the suitability of nanomaterials for introduction when performing screening allelopathic studies in a laboratory setting. In this aspect, the impact of eleven concentrations colloidal Nano Gold Gold-Rubin with nanoparticles 20 ppm on seed germination and initial development of *Lactuca sativa* L. variety Great Lakes and *Medicago sativa* L. variety Pleven 6 under laboratory conditions. It was found that applied higher concentrations (from 5.0 to 20.0 ppm) of colloidal Nano Gold in *L. sativa* and *M. sativa,* had an indifferent effect on the global germination index (GI) of the test plants, allowing concentrations of 20.0 ppm, to be used in performing allelopathic studies under laboratory conditions with both crops. However, a disproportionate influence on the biometric indicators of the test plants included in the study was found with increasing Gold-Rubin concentrations applied, suggesting the need for further research related to combined application with aqueous extracts or hydrolates of plants with proven allelopathic potential, including validation in vessel trials and greenhouse conditions.

Keywords: nanoparticles; nano solutions; inhibition; allelopathy; biotest

Introduction

Modern conventional agriculture requires high inputs of raw materials to increase the efficiency of growing crops (Tabaglio et al., 2008). According to the summarized studies of Légère et al. (2005), Oerke (2006) and Fernandez-Quintanilla et al. (2008), it was found that economic losses in conventional agricultural production are a result of weed infestation in production areas compared to the complex impact, caused by diseases and enemies. In a comprehensive analysis, Oerke (2006) summarized that weed infestation in agrophytocoenoses potentially limits losses in yields of agricultural production worldwide to 34%, while the complex effects of enemies and diseases determine yield losses of 18% and 16%, respectively.

Although at the modern stage in agriculture, conventional chemical weed killers (herbicides) with proven efficacy and rapid initial action are used to weed control in cultivated areas, intensive monocultural cultivation of agricultural crops requires the use of a limited range of active ingredients from the same groups to fight against weeds, which is a prerequisite for increasing the resistance of different weed species to different groups of herbicides, widely used in modern agriculture (Gaines et al., 2020; Carvalho-Moore et al., 2021; Hussain et al., 2021; Ofosu et al., 2023). All this necessitates the introduction, and use of environmentally friendly and innovative technological solutions from the modern concepts of minimally dosed anthropogenic impact in agrophytocenoses, to optimize weed density in cultivated areas (Swanton et al., 1999; Jabran et al., 2015; Mortensen et al., 2000; Bai et al., 2022).

At the modern stage in herbological practice, the phytocenological approach is being formed (Mirkin & Naumova,

2012), to limit the degree of weeding, that is, with minimal impacts and appropriate methods and techniques, sustainable development of cultural plants in agrophytocenoses is ensured, while the available weeds are kept under control level, below the biological and economic thresholds of harmfulness MacLaren et al. (2020).

In recent decades, a number of scientific studies (Duke, 2015; Mallik and Inderjit, 2002; Choudhary et al., 2023; Jabran, 2017; Khamare et al., 2022; Kostina-Bednarz et al., 2023), have emphasized allelopathy and the possibilities for inclusion, as an alternative to the herbicides widely used in agricultural practice, to limit weed species in agrophytocenoses. According to the authors, allelopathic relationships in plant communities determine the potential for ecological control in regulating the degree of weed infestation in modern agrophytocenoses, by using the phenomenal phenomenon – allelopathy, related to the synthesis and release of secondary metabolites (allelochemicals) from a number of plant species.

In a comprehensive analysis, Choudhary et al. (2023), Jabran et al. (2015), Alsaadawi et al. (2020), Singh et al. (2022), emphasize the allelopathic relationships in plant communities, and the potential opportunities to reduce the use of herbicides in cultivated areas, as a means of minimizing the concerns in modern society related to their incorrect application (in elevated doses), environmental pollution (accumulation of residues amounts in plant production, soil and groundwater) and to limit the resistance of a number of weed species to modern herbicides.

Despite established interrelationships in the dynamics of allelopathic relationships in plant communities, allelopathic interference is underutilized in agricultural practice due to prevailing limitations in unifying experimental practices related to the use of different carriers and/or extraction techniques of allelochemicals and their identification when performing screening studies in laboratory conditions (Bonanomi et al., 2006; Zhang et al., 2018; Singh et al., 2021; Hickman et al., 2023; Kato-Noguchi, 2024).

In the last decade, nanomaterials have been the subject of research by a number of researchers Kah et al. (2019), Mittal et al. (2020), Rajput et al. (2020), Vega-Vásquez et al. (2020), Grillo et al. (2021), Bandi et al. (2023), Gao et al. (2023), in relation to their application in agriculture, related to their use to improve plant growth in ontogenetic development, phytostatic effect to plant pathogens, as well as in plant protection practices, related to the migration and controlled release of agrochemicals in plants, ensuring the effective their use at reduced exposure.

In a comprehensive analysis, Siddiqi and Husen (2016), Mittal et al. (2020), Ferrari et al. (2021), summarize that considerable research attention has been focused on gold nanoparticles (AuNPs) due to their unique physicochemical properties – small size, large surface area to volume ratio, high carrier capacity and easy modification of their surface reactivity, which have a positive influence on the ontogenetic development and opportunities to increase the yield of agricultural plants.

When carrying out allelopathic studies in laboratory conditions, the screening methods for establishing allelopathic interference in plant communities ("weed – cultivated plant" and/or cultivated plant – cultivated plant), are most often used, which are based on establishing and proving the stimulating and/or inhibitory effect of extracted plant material (donor weed species or cultivated plants) with different (organic and/or inorganic), carriers on the germination and initial development of recipient test plants with proven high sensitivity to allelochemicals. The resulting extracts from plant fresh and/or dry biomass are extremely "unstable", creating suitable conditions for the development of microorganisms that have a negative impact on the germination and initial development of the recipient test plants.

In this regard, the use of nanosolutions with gold nanoparticles (AuNP) can probably be used as "carriers" of various biologically active substances (allelochemicals) in performing screening allelopathic studies without causing chemical-physical changes in them (Krishnaraj et al., 2010; Pudlarz and Szemraj, 2018; Sembada & Lenggoro, 2024). Despite their indisputable advantages, it has been found that some of the nanomaterials used can have a negative impact on the growth and ontogenetic development of plants, as well as induce phytotoxic changes (Nel et al., 2006; Barrena et al., 2009; Tiede et al., 2009; Taylor et al., 2014; Oliveri et al., 2020; Wang et al., 2023). According to Albanese et al. (2012), the bioavailability and cytotoxicity of nanoparticles are determined by multiple factors, and depend on their shape, size, concentration and mobility in an aqueous environment.

In a comprehensive analysis, Lin and Xing (2007) and Tarafdar et al. (2012) found that the use of nanomaterials had an indifferent to phytotoxic effect on seed germination and initial plant development. According to the authors, the observed phytotoxic changes on plants after the application of nanomaterials are more pronounced on the growth of root biomass compared to seed germination, which is limited and partially related to the size, shape and applied concentration of the nanoparticles used.

In this aspect, the aim of the study is to determine the effect of Gold-Rubin colloidal Nano Gold (AuNP) on seed germination and initial development of *Lactuca sativa* L. and *Medicago sativa* L., as well as its suitability for introduction in performing screening allelopathic studies in laboratory conditions.

Material and Methods

The experimental work was carried out in 2023 – 2024, under laboratory conditions. To establish the biological influence of Gold-Rubin colloidal Nano Gold with nanoparticles 20 ppm (>20 mg/l purity ≥99.9%), seeds of *Lactuca sativa* L. variety Great Lakes and *Medicago sativa* L. variety Pleven 6 were used – species with proven sensitive to potentially toxic substances (Shahriari et al., 2007; El-Kenany et al., 2017; Lyu et al., 2018; Wang et al., 2022a, b; Abdelmalik et al., 2024; Souzaa et al., 2024; Vieiraa et al., 2024), widely used as test plants in performing screening studies in laboratory conditions. The commercial product Gold-Rubin was applied at the equivalent of 100% initial concentration (20 ppm colloidal Nano Gold), and diluted with double-distillation water to final concentrations of 0.00, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5.0, 10.0 and 20.0 ppm

Semi-solid 0.75% agar-agar was used for development of the test plants included in the study (*L. sativa* and *M. sativa*). For this purpose, 0.45 g of agar was added to 60 ml of the applied concentrations of colloidal Nano Gold, after which they were tempered at 45°C. In Petri dishes (90 mm) 20 ml of 0.75% agar-agar were pipetted. After forming the agar gel, 50 seeds of *L. sativa* or *M. sativa* were placed on the media. Petri dishes were sealed with *Parafilm "M"* paraffin tape, placed in a thermostat (in the dark) at a temperature of 22 ± 2°C for five days. Agar-agar with double-distilled water was used as a control, at eight-fold repeatability for each variant.

To prevent microbial contamination of the agar gel from the seeds of the test plants, they were surface sterilized by placing them in micro-perforated textile silk bags, then immersed in a 0.2% v/v sodium hypochlorite solution for 5 min, followed by washing four times with double-distilled water, then immersed in 70% v/v ethanol for 2.5 min, and again washed four times with double-distilled water using a Büchner funnel (Li et al., 2017).

To establish the influence of the concentrations of colloidal Nano Gold included in the study on the germination and initial development of the test plants, the following indicators, indices and coefficients, presented in Table 1, were considered.

Table 1. Seed germination assessment parameters and the initial development of the recipient test plants of *Lactuca sativa* L. and *Medicago sativa* L.

Parameter/Reference	Formula	Explanation parameter
Percentage of germinated seeds, $GP_{\%}$ ISTA (2024), Wang et al. (2022)	$GP_{\%} = \left(\frac{NSG}{TNS}\right).100$	*NSG* – number of germinated seeds *TNS* – total number of seeds used in all experimental variants and replicates
Length of root, hypocotyl and seedling length, cm (SL) Golubinova et al. (2020)	$SL = \sum_{i=1\div50}^{n} I/n$	*I* – number of individual measurements of plant organs for all experimental variants and replicates *n* – number of all measurements
Fresh biomass of root, hypocotyl and seedling length, g (FB) Golubinova et al. (2020)	$FB = \sum_{i=1\div50}^{n} I/n$	
Percentage of inhibition germinated seeds, $I_{\%}$ Sundra & Pote (1978)	$I = 100 - (E_2.100/E_1)$	E_1 – response of plant seeds in the control E_2 – response of plant seeds from experimental variants At values of I% „+" – stimulating, „–" – inhibitory effect
Reduction of germinated seeds parameter, R Thabet et al. (2018)	$R = G_c - C_i$	Average values for biometric indicators of: G_i – experimental variants G_c – control (untreated) variant.
Log Response Ratio (LRR) Belter & Cahill (2015)	$LRR = \ln\left(\frac{V_n}{V_a}\right)$	V_a – mean level in control variant V_n – mean level in experimental variants
Global germination index, GI Gariglio et al. (2002)	$GI = \left[\left(\frac{G}{G_0}\right).\left(\frac{L}{L_0}\right)\right].100$	G and G_0 – germinated seeds in the experimental variants and the control (%); L – seedling length or fresh biomass in the experimental variants; L_0 – seedling length or fresh biomass in the control variant, taken as 100%

Raw data obtained were processed using the software products Statgraphics Plus for Windows Ver. 2.1 and Statistica Ver. 10, by one- and/or two-way factor analysis of variance analysis (ANOVA), using least significant difference (LSD), by Fisher's exact test at a confidence interval of 95% and error $\alpha = 0.05$. Percent germinated seeds (GP%) was transformed using (arcsin $\sqrt{x}$) (Ayeb-Zakhama and Harzallah-Skhiri, 2016). The power of influence of the factors was determined by η^2 with a reliable factorial variance of 99% (Plohinskii, 1967).

Results and Discussion

The commercial formulation included in the study, colloidal Nano Gold Gold-Rubin, had an indifferent stimulating effect on the laboratory seed germination of the test plants included in the study, with the difference, that it was relatively more pronounced in *L. sativa* compared to *M. sativa*. From the analysis of the data presented in Table 2, it is clear that the laboratory germination of the seeds of the species included in the study, does not increase proportionally with increasing the concentration of colloidal Nano Gold in the agar gel.

From the mathematical-statistical analyses of the experimental results, it is evident that regardless of the established differences in the reduction (R from 4.9 to 11.9 in *L. sativa* and from 2.8 to 8.9 in *M. sativa*), and the percentage of inhibition (I% from 7.2 to 17.2 in *L. sativa* and from 0.1 to 10.6 in *M. sativa*) in reported laboratory seed germination, the differences were not statistically proven (at ($p \leq 0.05$),

Table 2. Effect of applied concentrations of colloidal Nano Gold on germination of *Lactuca sativa* L. and *Medicago sativa* L. seeds

Variants				Indicators			
Test plants (Factor A)		Concentration, ppm (Factor B)		Percentage of germinated seeds, $GP_{\%}$	± standard error, SE	Reduction of percentage germinated seeds, R	Percentage of inhibition, $I_{\%}$
a_1	*Lactuca sativa* L.	b_1	0.00*	68.9ab	2.7	0.0	0.0
		b_2	0.04	57.0a	3.7	11.9	17.2
		b_3	0.08	57.0a	3.6	11.9	17.2
		b_4	0.16	57.0a	3.5	11.9	17.2
		b_5	0.31	57.0a	3.7	11.9	17.2
		b_6	0.63	63.9ab	4.3	4.9	7.2
		b_7	1.25	75.0bc	7.9	-6.1	-8.9
		b_8	2.5	83.9c	6.1	-15.0	-21.8
		b_9	5.00	57.0a	3.7	11.9	17.2
		b_{10}	10.0	63.9ab	4.2	4.9	7.2
		b_{11}	20.00	59.7ab	6.2	9.1	13.3
a_2	*Medicago sativa* L.	b_1	0.0*	83.9a	6.1	0.0	0.0
		b_2	0.2	83.9a	6.4	0.0	0.1
		b_3	0.4	83.9a	6.4	0.0	0.1
		b_4	0.8	75.0a	7.9	8.9	10.6
		b_5	1.6	81.1a	8.9	2.8	3.3
		b_6	3.1	83.9a	6.1	0.0	0.1
		b_7	6.2	83.9a	5.2	0.0	0.1
		b_8	12.5	90.0b	7.9	-6.1	-7.3
		b_9	25.0	75.0a	6.1	8.9	10.6
		b_{10}	50.0	83.9a	6.4	0.0	0.1
		b_{11}	100.0	83.9a	6.2	0.0	0.1

Table 2. Continued

Individual action of factors				
Factor A		GP%	Factor B	GP%
a_1	*L. sativa*	63.7a	b_1	76.4abc
a_2	*M. sativa*	88.1b	b_2	70.4ab
			b_3	70.4ab
			b_4	70.0a
			b_5	69.1ab
			b_6	73.9ab
			b_7	79.4bc
			b_8	86.9c
			b_9	70.0a
			b_{10}	73.9ab
			b_{11}	71.8ab
Influence of factors			*MS*	η^2
A			5892,0	44.9
B			225,7	17.2
AxB			69,6	5.3

Legend: *Control (double-distilled water); a, b, c, d – LSD at statistical significance at $p \leq 0.05$

compared with double-distilled water used as a control. An exception to the described dependence was found when applying colloidal Nano Gold in concentrations of 1.25 and 2.5 ppm in *L. sativa* and 2.5 ppm and in *M. sativa*, where a statistically proven ($p \leq 0.05$) stimulating effect was found in studies indicator. Analogous are the results, obtained when tracking the log response ratio (LRR) to determine the size of the effect of the applied concentrations of colloidal Nano Gold, on the laboratory germination of the seeds of the species included in the study (Figure 1).

From the data analysis, it is evident that the log response ratio (LRR) of *M. sativa* varies in a narrow range (LRR from 0.0 to +0.07 and from 0.0 to -0.11), while that of *L. sativa* is in the range of (LRR from 0.0 to +0.20 and from 0.0 to -0.19), which determines, from indifferent, to weak stimulation or inhibitory effect depending on the applied concentrations of colloidal Nano Gold on the laboratory germination of the seeds.

Regarding the independent action of the factors Factor A – the laboratory germination of the seeds of the *L. sativa* and *M. sativa* species included in the study, statistically proven differences ($p \leq 0.05$), are established, which can be explained by the differences in the anatomical-morphological seed characteristics, which Arora et al. (2012) and Zheng et al. (2005) are associated with an increase in the water permeability of the seed coat (spermoderm), facilitating seed hydration and di-oxygen into the cells, which accelerates the metabolism and germination process.

The applied concentrations of colloidal Nano Gold (Factor B) did not have a statistically proven effect ($p \leq 0.05$) on the laboratory germination of the seeds of the species included in the study. From the two-factor analysis of variance performed, to determine the weight of the factors (η^2) and the hierarchical distribution of variation in relation to the laboratory germination of the seeds of the species, included in the

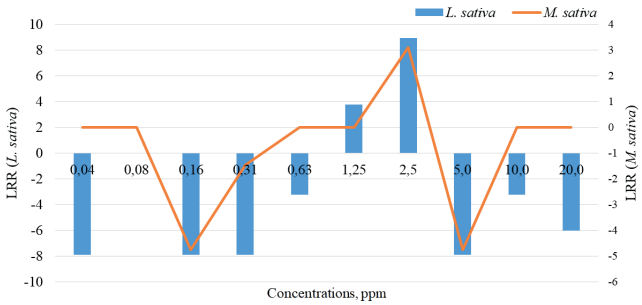

Fig. 1. Log response ratio (LRR) depending on the applied concentrations of colloidal Nano Gold on laboratory seed germination ($GP_{\%}$) of the species included in the study

study (Factor A), and the influence of the applied concentrations of colloidal Nano Gold (Factor B), it is established that relatively the largest share of the total variation is taken by Factor A (η^2 = 44.9%), followed by Factor B η^2 = 17.2%.

The values of the variances of the interaction of the studied factors AxB determine a relatively smallest and insignificant share of the total variation (η^2 = 5.3%). The obtained experimental results are in agreement with those reported by Asli & Neumann (2009), Arora et al. (2012), Mahakham et al. (2016), Parveen et al. (2016), Siddiqi and Husen (2016), Mahakham et al. (2017), Acharya et al. (2020), Song et al. (2022) according to which, the pre-sowing treatment of seeds of *Brassica juncea* (L.) Czern., *Cucumis sativus* L., *Gloriosa superba* L., *Lactuca sativa* L., *Medicago sativa* L., *Oryza sativa* L., *Pennisetum glaucum* (L.) R. Br. and *Zea mays* L. with AuNPs had an indifferent to positive effect on seed germination and initial plant development.

The data from the biometric measurements of root, hypocotyl and seedling length growth (cm) of the species included in the study, allows to compare and evaluate the influence of the applied concentrations of colloidal Nano Gold on the initial development of the test plants (Table 3).

From the data analysis, it is evident that a statistically significant ($p \leq 0.05$) increase in root, hypocotyl and seedling growth (cm) in *L. sativa* was recorded at the applied colloidal Nano Gold concentrations ranging from 0.63 to 2.5 ppm, while in *M. sativa* a stimulating effect on the studied indicators was found from 0.16 to 2.5 ppm.

Table 3. Influence of applied concentrations of colloidal Nano Gold on the initial development of *Lactuca sativa* L. and *Medicago sativa* L.

Variants length, cm				Indicators					
				length, cm			Fresh biomass per plant, g		
Test plants (Factor A)		Concentration, ppm (Factor B)		root	hypocotyl	seedling	root	hypocotyl	seedling
a_1	*Lactuca sativa* L.	b_1	0.00*	1.14ab	2.54ab	3.6ab	0.0015a	0.0098b	0.0113ab
		b_2	0.04	1.43ab	2.6ab	4.07a-d	0.0019b	0.0098b	0.0118b
		b_3	0.08	1.69bc	2.53ab	4.21a-d	0.0019b	0.0099b	0.0118b
		b_4	0.16	1.79bcd	3.57b	5.36b-e	0.0018b	0.0089a	0.0107a
		b_5	0.31	1.73bc	2.31a	4.04a-d	0.0021bc	0.0121e	0.0142d
		b_6	0.63	2.60e	3.00ab	5.60c-e	0.0022c	0.0106c	0.0129c
		b_7	1.25	2.14cde	3.46b	5.60de	0.0023c	0.0190g	0.0213f
		b_8	2.50	2.46de	3.61b	6.07e	0.0032d	0.0129f	0.0161e
		b_9	5.00	1.90de	3.06b	4.96b-d	0.0019b	0.0103bc	0.0122c
		b_{10}	10.00	1.30ab	2.53b	3.83a-c	0.0014a	0.0115d	0.0129c
		b_{11}	20.00	1.38ab	2.18a	3.49a	0.0015b	0.0098b	0.0113b
a_2	*Medicago sativa* L.	b_1	0.00*	3.65cd	5.21a	8.86ab	0.0080b	0.0228ab	0.0308ab
		b_2	0.04	2.06a	5.81ab	7.87a	0.0087bc	0.0230ab	0.0318a-c
		b_3	0.08	3.19bc	5.59ab	8.78ab	0.0087bc	0.0243b	0.0330b-c
		b_4	0.16	3.26b-d	6.74c	10.00c	0.0094c	0.0239b	0.0332b-c
		b_5	0.31	4.23d	6.30cd	10.53c	0.0101de	0.0261c	0.0361e
		b_6	0.63	3.75cd	6.64cd	10.39c	0.0106e	0.0272cd	0.0378g
		b_7	1.25	3.60cd	6.28a-d	9.88bc	0.0239f	0.0328d	0.0568f
		b_8	2.50	3.32b-d	6.57b-d	9.89bc	0.0086bc	0.0265c	0.0350de
		b_9	5.00	2.44de	6.94d	9.39a-c	0.0094cd	0.0222a	0.0317a-c
		b_{10}	10.00	3.20 b-d	5.70ab	8.90a-c	0.0055a	0.0241a	0.0297a
		b_{11}	20.00	2.56de	5.42ab	7.98a	0.0064a	0.0234ab	0.0299a

Table 3. Continued

Individual action of factors on growth, cm							
Factor A	root	hypocotyl	seedling	Factor B	root	hypocotyl	seedling
a_1	1.69a	2.87a	4.55a	b_1	2.40c-e	3.87a	6.27ab
a_2	3.21b	6.11b	9.32b	b_2	1.76ab	4.32a-c	5.94a
				b_3	2.48c-e	4.15ab	6.49a-c
				b_4	2.52 c-e	5.15c	7.68c-e
				b_5	3.11f	4.53a-c	7.38b-e
				b_6	3.27f	5.14c	7.70e
				b_7	2.84d-f	4.80bc	7.75de
				b_8	2.89ef	5.09c	7.98e
				b_9	1.72a	5.11bc	6.92a-d
				b_{10}	2.30b-d	4.20a-c	6.37ab
				b_{11}	2.00a-c	3.88a	5.79a

Influence of factors	root		hypocotyl		seedling	
	MS	η^2	*MS*	η^2	*MS*	η^2
A	229.150	33.5	1049.506	60.5	2264.72	59.3
B	9.044	13.2	9.551	5.5	25.49	6.7
AxB	3.294	4.8	1.608	0.9	5.32	1.4

Individual action of factors on fresh biomass formation in g per plant							
Factor A	root	hypocotyl	seedling	Factor B	root	hypocotyl	seedling
a_1	0.0020a	0.0114a	0.0133a	b_1	0.0047c	0.0163ab	0.0121ab
a_2	0.010b	0.0248b	0.0347b	b_2	0.0053d	0.0164ab	0.0218abc
				b_3	0.0053d	0.0171cd	0.0224cd
				b_4	0.0056de	0.0164ab	0.0220bc
				b_5	0.0061fg	0.0191e	0.0251e
				b_6	0.0064g	0.0169bc	0.0234d
				b_7	0.0131h	0.0259f	0.0391f
				b_8	0.0060ef	0.0197e	0.0256e
				b_9	0.0054d	0.0162a	0.0217abc
				b_{10}	0.0035a	0.0178d	0.0213ab
				b_{11}	0.0042b	0.0166abc	0.0208a

Influence of factors	root		hypocotyl		seedling	
	MS	η^2	*MS*	η^2	*MS*	η^2
A	0.00157	67.1	0.004455	80.9	0.011348	80.6
B	0.00006	24.5	0.000073	13.3	0.000246	17.5
A x B	0.00005	21.9	0.000002	0.3	0.000055	3.9

Legend: *Control (double-distilled water); a, b, c, d – LSD at statistical significance at $p \leq 0.05$

The indirect effect of applied concentrations of colloidal Nano Gold in the range of 0.04 to 0.31 ppm in *L. sativa* and from 0.04 to 0.08 ppm in *M. sativa*, as well as in concentrations of 5.0 to 20 ppm for both cultures, causing from a weak stimulation to a moderately inhibitory effect on the growth of the length of the root and hypocotyl, respectively, and of the seedling in the test plants, the reported absolute values being close to those recorded in the control variants and statistically unproven ($p \leq 0.05$).

Regarding the accumulation of fresh biomass in the root, hypocotyl and seedling generative organs of the test plants included in the study, a differentiated species reaction was found depending on the colloidal Nano Gold concentrations was used. The applied concentrations of colloidal Nano Gold

caused a stimulating effect on the accumulation of fresh root biomass (g) in *L. sativa* in the range of 0.04 to 5.0 ppm, while in the hypocotyl, respectively, and in the seedling it was in the range of 0.63 to 10, 0 ppm, while with *M. sativa* a stimulating effect on the studied indicators was found in a relatively narrow range from 0.16 to 2.5 ppm, with the differences being statistically proven to be increased ($p \leq 0.05$), compared to the control variants with double-distilled water. The lowest 0.04 and 0.16 ppm colloidal Nano Gold concentrations used, had an indifferent effect on the accumulation of fresh biomass in hypocotyls and seedling, respectively, in *L. sativa*, while in *M. sativa* the lower ones (from 0.04 to 0 .16 ppm), or the highest (5.0 and 20.0 ppm) concentrations used in the experiment, did not cause an increase in the formed fresh root biomass, hypocotyls and seedling, the reported absolute values being close to those, recorded in the control variants. The established differences in the stimulatory, and/or indifferent effect of the applied concentrations of colloidal Nano Gold in the reported absolute values related to the accumulation of fresh biomass in the generative organs (root and hypocotyl, respectively, seedling), of the *L. sativa* and *M. sativa* included in the study can be explained by specific species differences. Similar results were obtained in the experimental work of Alshehddi & Bokhari (2020), Ferrari et al. (2021), Bandi et al. (2023) according to which, the use of different concentrations of Nano Gold in a number of plant

Table 4. Log response ratio (LRR) depending on the applied concentrations of colloidal Nano Gold on the growth (cm) and formation of fresh biomass (g) in the generative organs (root, hypocotyl and seedling) of the *Lactuca sativa* L. and *Medicago sativa* L. species included in the study

Variants				LRR					
				length, cm			Fresh biomass per plant, g		
Test plants (Factor A)		Concentration, ppm (Factor B)		root	hypocotyl	seedling	root	hypocotyl	seedling
a_1	*Lactuca sativa* L.	b_1	0.00*	0.0	0.0	0.0	0.0	0.0	0.0
		b_2	0.04	0.227	0.023	0.123	0.236	0.000	0.043
		b_3	0.08	0.167	-0.027	0.034	0.000	0.010	0.000
		b_4	0.16	0.451	0.340	0.398	0.182	-0.096	-0.055
		b_5	0.31	0.417	-0.095	0.115	0.336	0.211	0.228
		b_6	0.63	0.824	0.166	0.442	0.383	0.078	0.132
		b_7	1.25	0.630	0.309	0.442	0.427	0.662	0.634
		b_8	2.50	0.769	0.352	0.522	0.758	0.275	0.354
		b_9	5.00	-0.236	0.186	0.095	0.236	0.050	0.077
		b_{10}	10.00	0.131	-0.004	0.062	-0.069	0.160	0.132
		b_{11}	20.00	0.191	-0.153	-0.031	0.000	0.000	0.000
		Average		0.424	0.127	0.250	0.298	0.158	0.179
a_2	*Medicago sativa* L.	b_1	0.00*	0.0	0.0	0.0	0.0	0.0	0.0
		b_2	0.04	-0.572	0.109	-0.118	0.084	0.009	0.032
		b_3	0.08	0.437	-0.039	0.109	0.000	0.055	0.037
		b_4	0.16	-0.113	0.257	0.121	0.161	0.047	0.075
		b_5	0.31	0.147	0.190	0.173	0.233	0.135	0.159
		b_6	0.63	0.027	0.243	0.159	0.281	0.176	0.205
		b_7	1.25	-0.014	0.187	0.109	1.094	0.364	0.612
		b_8	2.50	-0.095	0.232	0.110	0.072	0.150	0.128
		b_9	5.00	-0.403	0.287	0.058	0.161	-0.027	0.029
		b_{10}	10.00	-0.132	0.090	0.005	-0.375	0.055	-0.036
		b_{11}	20.00	-0.355	0.040	-0.105	-0.223	0.026	-0.030
		Average		-0.144	0.174	0.055	0.236	0.106	0.142

Legend: *Control (double-distilled water); a, b, c, d – LSD at statistical significance at $p \leq 0.05$

species caused a differential effect on the growth of root and aboveground biomass in the initial stages of the ontogenetic development of plants.

The established species-specific response in the initial development of the test plants depending on the applied concentrations of colloidal Nano Gold in terms of the changes in the biometric indicators related to the increase in length (cm) and the formed fresh biomass of the root, hypocotyl and seedling (g for one plant), were also defined by the evaluation of the power of influence of the factors (η^2). From the two-factor dispersion analysis performed to determine the hierarchical distribution of the variation depending on the weight of the factors (η^2) in relation to the studied biometric indicators, it is clear that relatively the largest share of the total variation is occupied by Factor A (the species included in the study) – η^2 is in the range from 35.5 to 80.9%, followed by Factor B (applied concentrations of colloidal Nano Gold, ppm) – η^2 is in the range from 5.5 to 24.5%. The values of the variances of the interaction of the studied factors AxB determine a relatively smallest and insignificant share of the total variation (η^2 – from 0.3 to 21.9%), which corresponds to their statistical significance ($p \leq 0.05$) in relation to their independent action (Table 3).

The results, obtained in monitoring the log response ratio (LRR) in determining the size of the effect of the applied concentrations of colloidal Nano Gold on initial growth (cm), and formation of fresh biomass (g) in the generative organs (root, hypocotyl and seedling) of the species included in the study (Table 4). Based on the results obtained and the analysis performed, it was found that the applied concentrations of colloidal Nano Gold have an indifferent effect (LRR=0) to an increased response (LRR "+") – stimulation in growth (cm), and to the formation of fresh biomass (g), or a negligible inhibitory effect (LRR "-") of the species included in the study. Regardless of the established differentiation of the species included in the study, the general trend of a stimulating effect as a result of the applied concentrations of colloidal Nano Gold on growth (cm) and on the formation of fresh biomass (g) in the generative organs (root and hypocotyl) in *L. sativa* and *M. sativa* (LRR on average ranges from +0.127 to +0.4324), respectively, and on seedlings (LRR on average – from +0.055 to +0.250) in both cultures. An exception to the described trend was found only in the increase of root biomass in *M. sativa*, where a weak to insignificant inhibitory effect was found (LRR average = -0.144) (Table 4).

The biological impact of the applied concentrations of colloidal Nano Gold can be expressed by a change in the morphological parameters of the test plants, in terms of average values of laboratory seed germination ($GP_{\%}$) and length (SL_{cm}), and/or formed fresh biomass of seedling (FB_g), by using global germination index (GI) (Figures 2 and 3).

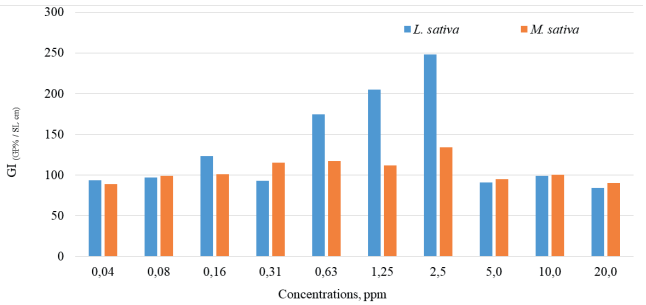

Fig. 2. The integral impact of colloidal Nano Gold on the global germination index (GI) of *Lactuca sativa* L. and *Medicago sativa* L. depending on the average values of laboratory seed germination ($GP_{\%}$) and seedling length (SL_{cm})

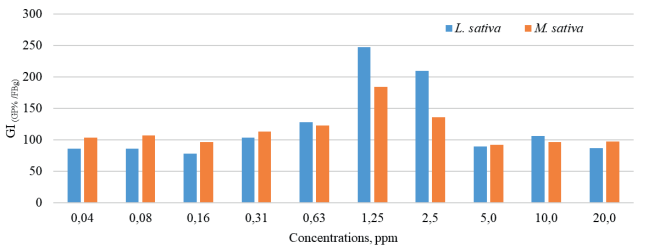

Fig. 3. The integral impact of colloidal Nano Gold on the global germination index (GI) of *Lactuca sativa* L. and *Medicago sativa* L. depending on the average values of laboratory seed germination ($GP_{\%}$) and formed fresh seedling biomass (FB_g)

The significant differences in the cumulative values of (GI), depending on the applied concentrations of colloidal Nano Gold, show a non-inhibitory effect GI≥80% (Zucconi, 1981) on the initial development of *L. sativa* $GI_{(GP\%/SL\ cm)}$ – from 84.0 to 248.2%; $GI_{(GP\%/FB\ g)}$ – from 78.3 to 248.0%, as well as for *M. sativa* $GI_{(GP\%/SL\ cm)}$ – from 88.8 to 134.0%; $GI_{(GP\%/FB\ g)}$ – from 92.0 to 184.4%. A stimulating effect on the test plants included in the study was found when colloidal Nano Gold was applied in concentrations from 0.63 to 2.5 ppm in the medium for initial development, as for *L. sativa* [$GI_{(GP\%/SL\ cm)}$ – from 174.4 to 248.2%; $GI_{(GP\%/FBg)}$ – from 128.0 to 248.0%], and for *M. sativa* [$GI_{(GP\%/SL\ cm)}$ – from 111.5 to 143.0%; $GI_{(GP\%/FB\ g)}$ – from 122.7 to 184.4%]. Applied lower (from 0.04 to 0.31 ppm) or higher concentrations (from 5.0 to 20.0 ppm), did not have a stimulating effect on the initial development of the test plants.

Based on the obtained experimental results and the summary conclusions of Zhu et al. (2012), Li et al. (2016), Dykman and Shchyogolev (2018), it can be generalized that the use of colloidal Nano Gold can find application in performing screening allelopathic studies in laboratory conditions, since according to the authors the efficiency of tissue penetration of gold nanoparticles is very high, they absorb well only from the root biomass and can efficiently move in the plants, which is a prerequisite for their use as a carrier of allelochemicals.

Therefore, the addition of Gold-Rubin (colloidal Nano Gold with nanoparticles) at concentrations of 5.0 to 20.0 ppm is likely to increase the total surface area of the allelochemical donor in semi-solid agar as a growth medium for *L. sativa* and *M. sativa,* which suggests better migration and/or contact of allelochemicals with the test plants, will help to establish and/or increase the allelopathic effect on the recipient test plants when performing screening studies, to establish the allelopathic potential of weed species and/or crop plants in laboratory conditions.

Conclusions

Following the applied experimental approach, an *in vitro* test was developed to optimize the approaches when performing allelopathic studies in laboratory conditions, by adding colloidal Nano Gold in redistilled water, as a possible carrier of allelochemicals.

It was found that applied higher concentrations (from 5.0 to 20.0 ppm) of colloidal Nano Gold Gold-Rubin in redistilled water to the growth medium (agar-agar) of *Lactuca sativa* L. and *Medicago sativa* L., showed an indifferent effect on the global germination index (GI) of the test plants. Concentrations of 20.0 ppm of colloidal Nano Gold can be used in performing allelopathic studies under laboratory conditions with both crops.

Further research is needed to establish the impact of colloidal Nano Gold when combined with aqueous extracts or hydrolates of plants with proven allelopathic potential, including validation in greenhouse conditions.

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