SCIENTIFIC REPORT

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Project title: **The use of engineered nanomaterials in plant science (*NanoPlant*)**

Contractor: **Universitatea de Ştiinţe Agricole şi Medicină Veterinară „Ion Ionescu de la Brad" Iaşi**

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Stage 2	**Synthesis and characterisation of engineered metal nanoparticles (part II). Study of the NPs influence on plants growth under controlled environmental conditions (part II) and in field conditions. Investigation of the antifungal properties of NPs on soil diseases (part I).**	**255.155,00 lei**
Activity 2.1	Optimum conditions identification for metal nanoparticles engineering by chemical, electrochemical or sonochemical synthesis (finalization)	
Activity 2.2	Nanophase and structural properties characterization of nanoparticles in solution and in film by AFM, SEM, XRD, FTIR, UV-VIS, Zeta potential, etc. (finalization)	
Activity 2.3	Investigation of the NPs effects on bulbs development under controlled conditions	
Activity 2.4	Identification of the optimum type and concentration of nanoparticles applied to plants	
Activity 2.5	Administration of nanoparticles to seeds of plants and bulbs that germinate in vegetation vessels	
Activity 2.6	Plants transplantation in the field after germination	
Activity 2.7	Assessment of the influence of nanoparticles on the development of plants under real growth conditions	
Activity 2.8	*In vitro* studies of the antifungal properties of nanoparticles	

The scientific objectives of this stage have been completely realized: the optimal conditions for obtaining nanoparticles have been identified and different types of nanoparticles were synthesized and characterized. Subsequently, the effects of the synthesized nanoparticles on seed germination were investigated.

Studies conducted at this stage of the project are very important in the current international context of research regarding the use of nanoparticles in plant science. Therefore, the value and scientific level of the results obtained so far are high and there are prerequisites for valuable results that can be published in the next period.

Synthesis of gold nanoparticles stabilized with chitosan

The optimization of the size of the gold nanoparticles (AuNPs) was based on the concentration of $HAuCl_4x3H_2O$ precursor and the energy injected into the ultrasonic field system. AuNPs were prepared in chitosan solutions of different molecular weights, studying the role as reducing and stabilizing agent of chitosan depending on its mass. The homogenization and thermo-precipitation process was accomplished by treating the initial solution with an ultrasonic field.

Precursor stock solutions of $HAuCl_4$ (0.01 M) and 0.1% chitosan in 0.5% acetic acid were prepared. These were mixed in a different ratio so as to obtain AuNPs solutions of different concentrations (25, 50, 75 μg / ml), and then were left under the influence of an ultrasonic field for 20 minutes at a temperature of 55°C. AuNPs formation has been demonstrated, at first view, by the coloring of the solution (Figure 1) after two days (the original solution being transparent). The coloration is closely related to the size of the Au nanoparticles, the geometric shape, the polymer mass covering the nanoparticles, etc.

Fig. 1. AuNPs in chitosan solutions (different concentrations)

It has been found that for all types of chitosan, high amounts of $HAuCl_4$ determine concentration gradients that lead to the creation of gold microcrystallites forming a brown precipitate, the solution with residual amounts of nanoparticles being yellow. Precipitation also occurs at lower concentrations of chitosan with a low molecular weight.

Synthesis of citrate-stabilized gold nanoparticles

The optimum conditions for the production of medium gold nanoparticles, stabilized with citrate, were established and their synthesis by the Turkevich method was studied. The influence of the various parameters (molar ratio of sodium citrate:chloroauroic acid, temperature of the reaction mixture, reactant concentrations) on nanoparticle size and shape to obtain high stability, low polydispersity and, implicitly, reproducible results was considered. Thus, an aqueous solution of $HAuCl_4$ (0.21 mM) was heated to 60°C with vigorous stirring, and then an aqueous

solution of pre-heated sodium citrate (1.3 mM) was added. The mixture was stirred for two hours at 85°C. Colloid formation is indicated by the change in the initial color of the solution from pale yellow to red. The solution was allowed to cool to room temperature, and then stored at 4°C.

In order to optimize the synthesis process, several attempts were made in which the precursor solution of $HAuCl_4$ was heated to temperatures in the range of 50-100°C. However, optimal results related to the quality and stability of AuNPs were obtained for an initial temperature of the precursor solution of 60°C.

Quantum dots carbon nanoparticles generated by laser ablation in liquid

Using liquid laser ablation, stable carbon nanoparticles (NPC) suspensions in ethanol were obtained without the use of surfactants. A target of pyrolitic graphite (99.99% purity) in disk form (thickness 2 mm) was immersed in 15 ml of ethanol (99.99%, Sigma Aldrich) and subjected to the incidence of a Nd:YAG laser beam (wavelength 1064 nm, pulse duration 7 ns) focused with a spherical lens (focal length 15 cm). The experimental arrangement allows the change of height of the liquid column without affecting the focal focus lens distance and changing the laser fluency by shifting the focusing lens relative to the target position.

Also, to ensure the reproducibility condition and increased nanoparticle production rate, the laser spot was shifted to a helical trajectory on the target surface using a linear displacement system of the mirror-lens assembly in the direction of propagation of the laser beam in a plane parallel to the plane of the target, concomitant with a rotation movement of the target around its own axis. NPC solutions were obtained for two laser fluency values of 2 and 3 J/cm^2, resulting the samples named C-Dot (F1) and C-Dot (F2), respectively.

Investigation of the NPs effects on seed germination of root vegetables (carrot), Solanaceae (tomato) and bulbous vegetables (onion) under controlled conditions

Germination is an important indicator in the appreciation of seed quality. It is carried out in a variable period of time, being influenced by a number of factors, among which the species and treatment applied to the seeds are of great importance.

In the first stage of the **NanoPlant** project, the influence of nanoparticle solutions treatments on seed germination was studied to identify optimal nanoparticle solutions, their concentration, duration of treatment, and the type of nanocomponent incorporated into the solution to stimulate the seed germination process. The experimental protocol consisted in the organization of a bifactorial experiment using three species of vegetable seeds that were subjected to a series of treatments with nanoparticle solutions. The experiment was performed for three batches of samples (three repetitons), and a control sample set, treated with either distilled water

or 0.1% chitosan, for each batch.

The bifactorial experiment was based on:

Factor A - Species used, with three graduations:

a_1 – carrot seeds, *Daucus carota* convar. *sativus* (Alef.), *Umbelliferae* fam.

a_2 – onion seeds, *Allium cepa*(L.), *Liliaceae* fam.

a_3 – tomato seeds, *Lycopersicon esculentum* (Mill.), *Solanaceae* fam.

Factor B - seed treatment, with nine graduations:

b_1 – AuNPs + chitosan (50 µg/ml)

b_2 – AuNPs + chitosan (75 µg/ml)

b_3 – AuNPs + citrate (2.6 mM)

b_4 – AuNPs + citrate (5.2 mM)

b_5 – C-Dot (F1)

b_6 – C-Dot (F2)

b_7 – chitosan 0.1%

b_8 – H_2O

b_9 – AuNPs + citrate (2.6 mM) - 24 ore

For all three studied species untreated seeds were used and a single cultivar was chosen for each species:

- carrots - "Lunga di Nantes" variety produced by Raci Sementi - Italy, Adri Prodcom S.R.L. importer;
- onion - "Dorata di Parma" variety produced by Raci Sementi - Italy, Adri Prodcom S.R.L. importer;
- - tomatoes - "Marisa" hybrid produced by Monsanto Holland BV - Netherlands, Universal Impexsem Co. S.R.L. importer.

Selected seeds were treated with different solutions of nanoparticles, of different concentrations and for a different interval of period. Consequently, the second factor in the experiment, factor B, presents nine graduations:

- variant 1 – AuNPs + chitosan (50 µg/ml): the seeds were soaked into the solution for 2 hours, noted variant **S1**;
- variant 2 – AuNPs + chitosan (75 µg/ml): the seeds were soaked into the solution for 2 hours, noted variant **S2**;
- variant 3 – AuNPs + citrate (2.6 mM): the seeds were soaked into the solution for 2 hours, noted variant **S3**;
- variant 4 – AuNPs + citrate (5.2 mM): the seeds were soaked into the solution for 2 hours, noted variant **S4**;

- variant 5 – C-Dot (F1): the seeds were soaked into the solution for 2 hours, noted variant **S5**;
- variant 6 – C-Dot (F2): the seeds were soaked into the solution for 2 hours, noted variant **S6**;
- variant 7 – chitosan 0,1%: the seeds were soaked into the solution for 2 hours, noted variant **S7**;
- variant 8 – H_2O (distillated water): the seeds were soaked into the solution for 2 hours, noted variant **S8**;
- variant 9 – AuNPs + citrate (2.6 mM): the seeds were soaked into the solution for 2 hours, noted variant **S9**.

In this report, with M were noted the samples for carrot, C for onion, and T for tomato.

After application of the treatment according to the scheme above, the seeds were placed in Petri dishes, on filter paper wetted with distilled water. In each Petri dish there were arranged 30 seeds, ordered as evenly as possible, on 6 lines for a easy evaluation of their germination, but also for providing sufficient space for the development of plants in germination and initial growth phases.

During the experiment, the Petri dishes were kept in the germinator (SANYO, Model MLR-351H), where the environmental parameters were controlled (Figure 2):

- the temperature was set at 16ºC – during daytime (6:00 - 21:00) and 13ºC - for night (21:00 - 6:00),
- humidity was constantly kept at 80%,
- light. The illumination of the samples was done with white light in monochrome radiation of 2500 lux between 6:00 and 21:00, and during the night it was reduced to 0 lux.

During the experiments, the seeds were maintained in optimum moisture conditions, favourable to germination. Thus, watering with distilled water was carried out whenever necessary to maintain the same moisture of the seeds.

Fig. 2. The germinator used and the germination conditions

The experiment was monitored throughout the day, with daily observations, with all data on seed germination being collected. Since the embryo broke the seed skin, observations and determinations have been made on the dynamics of the daily germination until at least two consecutive determinations have revealed the same number of germinated seeds. The total percentage of seeds germinated each day for each variant was noted, as well as two other parameters: **the germination rate** and **the germination velocity**. These parameters are necessary for a more objective evaluation of the effects of the applied treatments.

The germination rate is the percentage of plants germinating each day or the percentage of plants germinated from one day to the next.

The germination velocity is the percentage of plants germinated in the time unit (day) and is calculated by the formula:

$V_G = \frac{Gi}{n}$, where:

Gi = germination at a given time;

n = the number of days in which the germination (Gi) occurred.

Analysis of plant sizes during germination phenophase

The study of the influence of nanoparticles on the physiological process of germination of vegetable seeds involves the analysis of plant sizes during germination and initial growth phenophases, measured on the last day of determination.

Carrot

The individual results on plant sizes in the first growth phases are correlated with the evolution of seed germination. However, the average results are slightly different, the MS6

variant presenting the higher dimensions and the highest error. The error results from the high unevenness of germination rates (Figure 3).

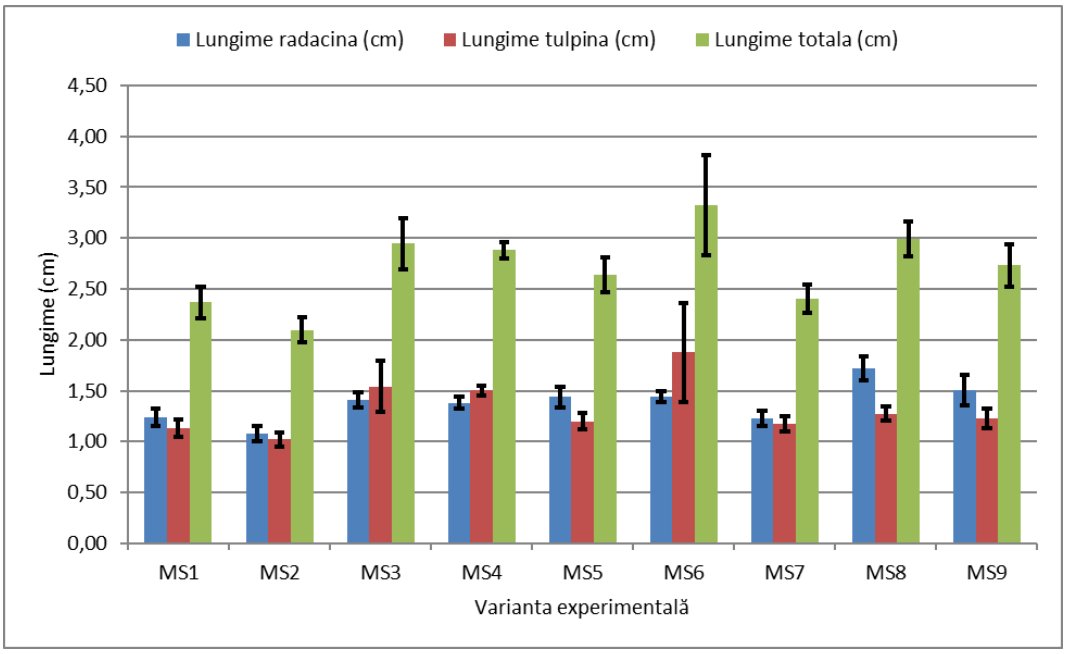

Fig. 3. Graphic representation of the dimensions of carrot plants at the end of the experiment

The smallest error was recorded for the variant MS4, which had a germination value of 88%. Another variant that responded well to the treatment was MS5, with a small error in plant size and the highest final germination (90%).

Onion

Individual results on the size of the plants in the germination and initial growth phenophases for onions are correlated with the development of seed germination for all variants, except CS9 that presents large errors (Fig. 4).

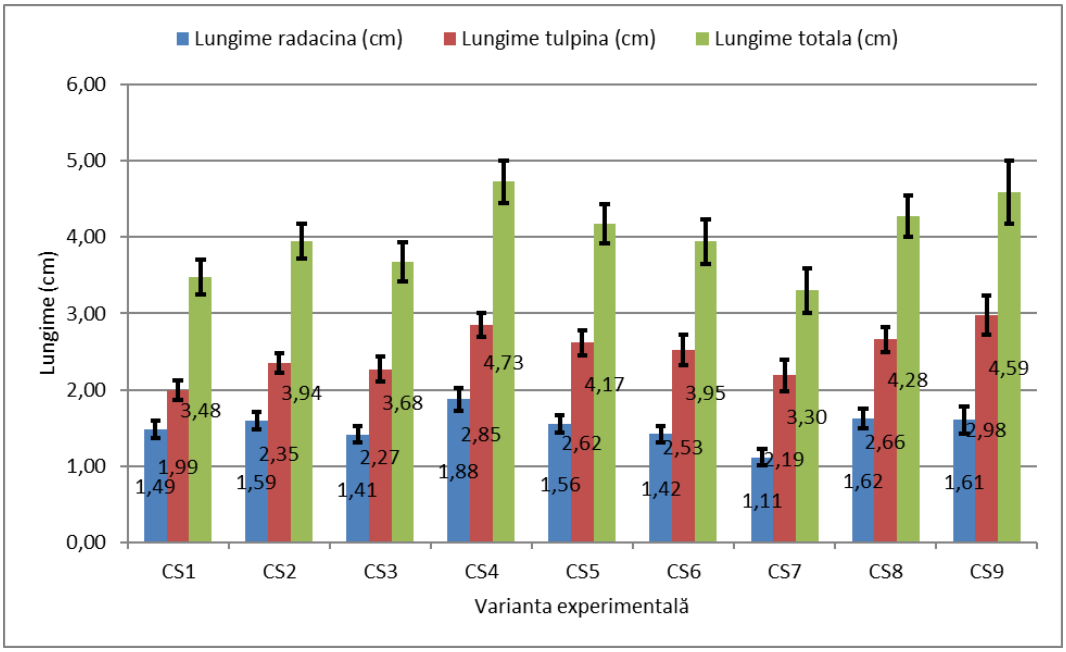

Fig. 4. Graphic representation of the onion plants dimensions at the end of the experiment

For onions, the smallest errors were recorded in the case of the CS1 variant, for which a high germination value (93%) was recorded and in the case of the CS2 variant, for which the final germination value was 97%. Therefore, these two variants present not only a high germination rate, but also uniformity in the initial growth phenophase.

Tomato

In the case of tomatoes, the environmental conditions and applied treatment inhibited the germination process. From Figure 5 it can be seen, as expected from the evaluation of germination, that the errors are very high, and in this case the results are inconclusive.

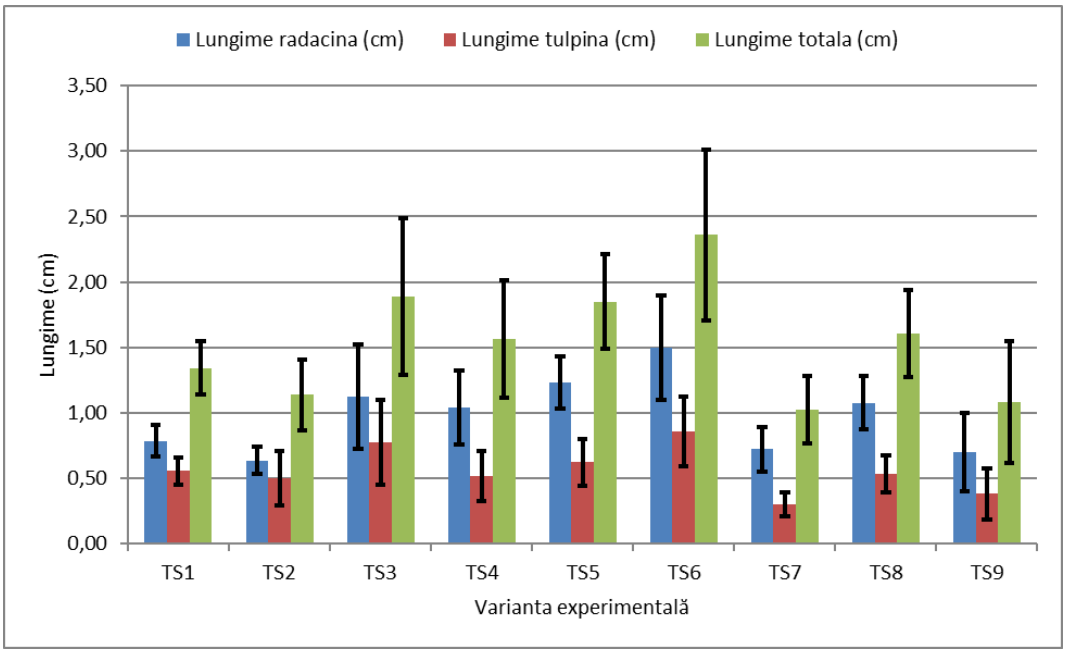

Fig. 5. Graphic representation of the size of tomato plants at the end of the experiment

Conclusions

The experimental results presented above show that the carrot and onion seeds react differently to the applied treatments, but with generally positive results for medium-term (two-hour) treatments.

In the case of long-term treatments (seeds soaked in nanoparticle solutions for 24 hours), they exhibit germ inhibition. The variant 9, both in carrot and onions, determines poor results, which are manifested by unevenness in germination in the case of carrot seeds and low germination in the case of onion seeds.

Following the treatments for carrot, the MS4 and MS5 variants have been found to have a stimulated effect on germination and uniformity in increases in the first phenophases. For onions, the CS1 and CS2 variants present high germination (93% and 97%, respectively) and uniformity in the initial growth phenophase.

For tomato seeds, the experiment must be repeated under different environmental conditions, especially by increasing temperature.