Determination of the Effect of Magnesium Oxide Nanoparticles (MgO-NP) on *in Vitro* Culture of Cowpea (*Vigna Unguiculata* L. Walp)

Rabia KOÇAK

Ataturk Universitesi Ziraat Fakultesi

Melih OKCU (✉ melihokcu@atauni.edu.tr)

Ataturk Universitesi Ziraat Fakultesi <https://orcid.org/0000-0001-5213-2169>

Kamil Haliloglu

Ataturk Universitesi Ziraat Fakultesi

Azize Alayli

Sakarya University: Sakarya Universitesi

Hayrunnisa Nadaroglu

Ataturk University: Ataturk Universitesi

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Abstract

This study was carried out to determine the effect of different doses of MgO nanoparticles (MgO-NP's) on *in vitro* regeneration of cowpea. MgO NPs used in the study were synthesized using walnut shell extract by green synthesis method. MgO nanoparticles with 35-40 nm size were used in this research. In the study, the effect of different doses of MgO NP applied to cowpea plant on all in vitro parameters was found to be significant.

Considering the parameters examined, the best results in morphogenesis were 185 mg/L, 370 mg/L and 555 mg/L MgO-NPs applications, the highest shoot formation rate was obtained 82.50% with 555 mg/L (MgO-NPs) and 72.50% with 370 mg/L (MgO-NPs). The highest mean values of shoot number, shoot number per explant and shoot length were observed in the application of 370 mg/L MgO-NPs with 61.25, 17.50, and 2.075 (unit) respectively.

The best root formation rate was obtained from control and 370 mg/L MgO application at a rate of 27.50%. The highest values were 6.75 (pieces) and 370 mg/L MgO-NPs in the number of roots per explant, 555 mg/L MgO-NPs with 10% in callus formation, and 1.575 (cm) and 370 mg/L MgO-NPs in root length. When all the examined parameters were evaluated, it was determined that the application of 370 mg/L MgO-NPs gave the best results in terms of in vitro parameters.

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a very common annual plant, especially in Africa, South America, Asia and the United States, and is one of the most important legumes worldwide (Xiong et al. 2016). It is a good pre-plant that has the ability to grow in poor soils and increases the yield of the next product with the help of nitrogen fixation (Miller et al. 1984; Pemberton and Smith 1990). Cowpea, which is considered as a green vegetable and dry grain in human nutrition and as a fodder in animal nutrition, belongs to the legume family and contains 2.0-4.3% protein in fresh beans and 4.5-5.0% in fresh grains. Protein content in dry cowpea grains that have reached maturity varies between 20.42-34.60% depending on the variety and environmental conditions. In addition, its grains contain 50-67% carbohydrates, 1.3% oil, 3.9% cellulose and 3.6% ash (Vandemark et al. 2014). The protein in its seeds is rich in Lysine and Tryptophan amino acids compared to cereal seeds, and insufficient in terms of Methionine and Cystine compared to animal proteins (Davis et al. 1991).

Nanotechnology, which is a multidisciplinary science, is a science that controls nano-sized materials and includes science and technology (Arnall 2003). In other words, it is defined as the existence of substances smaller than 100 nanometers in size and controlling them at the atomic level and making them useful (Bergeson 2004). Nanotechnology, which is an interdisciplinary research field developing in many different fields, covers pesticide distribution, nanosensors, pesticide degradation, use of micronutrients in agriculture, plant protection and nutrition, agricultural applications (Ghormade et al. 2011). Nanotechnology offers effective methods to protect soil health and conditions by helping to minimize agricultural waste and environmental pollution (Raliya et al. 2017; Duhan et al. 2017). Nanotechnological approaches can greatly improve the functioning of precision agriculture (Panpatte and Jhala 2019).

Magnesium oxide is an important inorganic material with a wide band range (Al-Gaashani et al. 2012). This material is used in many applications such as catalysis, catalyst supports, toxic waste reclamation, refractory materials and adsorbents, additive in heavy fuel oils, reflective and anti-reflective coatings, substrate such as superconducting and ferroelectric thin films, superconductors and lithium-ion batteries (Ouraipryvan et al. 2009; Mirzaei and Davoodnia 2012). Nano MgO, on the other hand, has many special physical and chemical properties brought about by its nano size. With its size, nanoparticles can be used more by plant cells, induce plant growth, and have antimicrobial, antifungal and antiviral effects against pests.

Magnesium, which plays an important role in plant development and physiology, is a component of the synthetic chlorophyll pathway that regulates the activity of key photosynthetic enzymes in the chloroplast (Sawicki et al. 2017). Magnesium deficiency suppresses plant growth and decreases yield (Stagnari et al. 2011). Magnesium is a macronutrient that activates more enzymes than other nutrients (Epstein and Bloom 2005) and has structural and regulatory functions related to nucleophilic ligands in plants (Shaul 2002; Cakmak and Kirkby 2008). It is one of the essential elements in the function and synthesis of nucleic acids and ATP (Sreedhara and Cowan 2002; Igamberdiev and Kleczkowski 2015).

Nanotechnology is useful in the agricultural sector, in the form of nanopesticides and nanofertilizers. In addition, using this branch of science, the synthesized nanomaterials provide protection that is effective in controlling pests and pathogens that significantly affect the yield of the plant (Prasad et al. 2017). Wide applications of NPs in plant tissue culture include the elimination of microbial contaminants from explants, callus induction, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation and secondary

metabolite development. Compounds can be developed by integrating the concept of nanotechnology into plant tissue culture techniques, synthesis, purification and desired plant-derived yield. Such approaches could have important industrial applications, as many phytochemicals are used as medicinal products.

Investigation of the effect of nanoparticles on in vitro propagation characters on cowpea plant, which has a great importance in human and animal nutrition, is a study that is lacking in the literature. In this context, it is aimed to determine the possible effects of callus formation on morphogenesis and plant regeneration by applying different doses of MgO NPs of cowpea plant.

Material And Method

Synthesis of Mg Nanoparticles (MgO-NPs):

MgO NPs were synthesized using walnut shell extract. The walnut shell extraction used for green synthesis was prepared with distilled water, and for this purpose, 25 g walnut shells were washed and crushed using the freeze-thaw technique in liquid nitrogen, 250 mL of distilled water was added and mixed in a magnetic stirrer for 1-2 hours. Then, the extract obtained by first filtering through cheesecloth and then filter paper was kept at -25 °C until use. 0,1 M $\text{Mg}(\text{NO}_3)_2$ çözeltisi bitkilerde MgO dönüşümü için aktif bir bileşik olarak kullanılmıştır. The formation of the synthesis was followed qualitatively and quantitatively by UV-Vis spectrophotometer. After the method optimization, the obtained nanoparticles were characterized. For this purpose, different morphological and molecular detection methods (SEM, FT-IR, XRD) were used. The obtained MgO-NPs were washed under vacuum using distilled water and ethanol and used in the reaction after drying in an oven. In the application range, after MgO-NPs were weighed and homogenized in pure water with the help of an ultrasonicator, they were used in plant in vitro experiments (Nadaroglu et al. 2017; Gultekin et al. 2017).

Plant Material

The cowpea cultivar, registered as "Ülkem" used in this study was obtained from Ondokuz Mayıs University and usually used for forage purposes. Forage cowpea seeds subjected to surface sterilization were kept in water overnight and the plumule parts of the embryos were removed and used as explants in the experiment.

Tissue Culture Applications:

MS medium, mineral salts and vitamins (Murashige and Skoog 1962) were used in the experiments. Magnesium and MgO-NPs in MS medium were exchanged with different concentrations. In the experiment, there were 5 treatments having different MgO NPs content. MS medium without MgO NPs in MS medium (control), amount of MgO NPs (1X - 370 mg/L), 0.5X (185 mg/L) MgO-NPs, 1X (370 mg/L) MgO-NPs and 1.5 X is (555 mg/L) MgO-NPs. Plumules were cultured on MS mediums containing MgO-NPs previously prepared. The explants were kept in the dark at $24\pm1^\circ\text{C}$ in callus initiation medium (MS salt and vitamins + 1.0 mg/L BAP) containing different Mg type and concentration for 4 weeks, and then they were placed in regeneration medium (MS salt and vitamins + 0.5 mg/L BAP) containing different MgO NPs type and concentration and kept under white fluorescent light (Preheat Daylight-42 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) for 4 weeks at $24\pm1^\circ\text{C}$ in a 16-hour light photo period. Morphogenesis, callus formation and rate measurements were made after the first 30 days. The number of shoots, shoot formation rate, shoot length (mm), shoot number per explant, root formation rate, number of roots per explant and root length (mm) parameters were measured after the second 30 days.

This study was carried out in complete randomized experimental design in factorial arrangement with four replications. Each petri dish was considered as an experimental unit and 10 cowpea plumula were cultured in each petri dish. Analysis of variance and Duncan multiple comparison test were computed with SPSS statistical analysis program (Version 20).

Results And Discussion

Surface morphological characterization of MgO NPs

Surface characterization of MgO NPs was performed using SEM, FTIR and XRD analyzes and the results are given in Figures a,b and c. Chemical and mineralogical compositions of MgO NPs obtained by green synthesis method were determined using Metek, Apollo prime,

Active area 10 mm², Microscope examination S50, SE detector R580. SEM analysis determined that the MgO-NPs obtained by green synthesis were well dispersed and cubic (Figure 1A).

While the peaks at 39.2° (111), 62.53 (220), 77.8° (311) and 81.7° (222) 2θ° in the XRD graph in Figure 1B belong to Mg(OH)₂. The diffraction peaks are points represent cubic MgO-NPs at 42.76° (200)2θ and 62.6° (220)2θ (Nguyen et al. 2021).

FT-IR analysis is an effective technique used to identify possible peaks of MgO NPs and extract used for reduction of metal. FT-IR analysis of MgO NPs obtained by green synthesis is given in Figure 1C. As seen from the FTIR diagram, the wavelength between 400 and 4000 cm⁻¹ was scanned. From the findings, it was determined that intense absorption peaks occurred at 3699, 3351, 2293, 1600, 1354, 1014, 763 and 519 cm⁻¹. While the peak observed at 3699 cm⁻¹ belongs to the -OH band, the wide peak band observed at 3351 cm⁻¹ indicates the presence of -NH₂ and -OH groups in the medium. The peaks seen around 1600 cm⁻¹ indicate the presence of peaks defined as the primary amine group (N-H) overlapping with the amide and carboxylate group. The peak of the peak at 1354 cm⁻¹ is matched with the Mg-OH group, while all bands between 400-736 confirm the presence of MgO NPs. The size of MgO nanoparticles was determined to be 35-40 nm as a result of measurements and calculations. The obtained spectrum showed that the walnut shell extract had a high ability to reduce and stabilize MgO NPs. Similar findings were found in the FT-IR analyzes of MgO NPs obtained using some plant extracts in the literature, and they support our study (Amina et al. 2020; Asami et al. 2016; Dobrucka 2018; Karimi et al. 2015; Saied et al. 2021; Somanathan et al. 2016; Suresh et al. 2018).

Morphogenesis

The averages of the characters determined by the treatments of different concentrations of magnesium nanoparticles to the cowpea and the related variance analysis results are given in Table 1.

Table 1
Average values of the parameter ratios of magnesium nanoparticles at different doses examined in cowpea plant

Magnesium Nanoparticle Applications	Morphogenesis	Shoot Formation Rate (%)	Number of shoots (pieces)	Number of Shoots per Explant (piece)	Shoot length (cm)	Root Formation Rate (%)	Number of roots per explant (piece)	Callus Formation	Root Length (cm)
Control	9.25	60 ab	21.75 b	6.50 bc	0.4750 d	27.50	2.750000 b	8 b	1.075000 b
370 mg/L MgO-NPs	9.25	42.50 bc	21.75 b	6.75 bc	1.2000 c	27.50	6.750000 a	9 ab	1.575000 a
185 mg/L MgO-NPs	10	30 c	3.75 b	1.25 c	0.1750 d	19.75	0.000900 c	8.5 ab	0.000150 c
370 mg/L MgO-NPs	10	72.50 a	61.25 a	17.50 a	2.0750 a	22.50	0.750050 bc	9.50 ab	0.275075 bc
555 mg/L MgO-NPs	10	82.50 a	36.25 ab	10.00 ab	1.4500 b	10.00a	0.750050 bc	10.00 a	0.20000 bc
Mean	9.7	57.50	28.95	8.40	1.0750	21.45	2.200028	9.00	0.625045
Variation Sources	SD	F Values							
Replication	4								
Magnesium Nanoparticle Applications	4 1,350*	1837,5**	3,459*	5,675**	88,901**	1,905*	18,030**	2,679*	20,402**
Error	15								
Acknowledgements									

These changes began to be observed after the first week of culture initiation. While the average number of explants showing morphogenesis was 9.25 at control (0 mg/L Mg) and 370 mg/L doses, this number was decreased in Mg-Np applications (185 mg/L,

370 mg/L and 555 mg/L MgO-NPs). The number of morphogenesis was 10. A significant 8.11% increase ($p<0.05$) in the number of morphogenesis was observed in 185 mg/L, 370 mg/L and 555 mg/L MgO-NPs applications compared to the control.

Morphological changes such as tissue growth and tissue swelling were observed in the cultured cowpea explants (Figure 2; A, B).

Callus formation:

It has been observed that different MgO-NPs applications have significant effects on callus formation. In the study, the average callus formation determined according to magnesium nanoparticles in cowpea varied between 8 and 10. Callus formation reached the highest value with 10 units in the application of 555 mg/L MgO-NPs. This application was followed by an average of 9.5 to 370 mg/L MgO-NPs and 9 to 370 mg/L MgO applications. Different Mg treatments significantly increased callus formation compared to control. The highest increase was observed with 25% in 555 mg/L MgO-NPs application, followed by 18.75% with 370 mg/L MgO-NPs, 12.50% with 370 mg/L MgO and 6.25% with 185 mg/L MgO-NPs applications (Table 1).

Shoot Formation:

Different MgO-NPs applications (with or without NPs) created significant differences in shoot formation ($p<01$). According to the effect of different doses of magnesium nanoparticles applied to the cowpea, shoot formation rates varied between 20.83% and 37.50%. The highest value in shoot formation rate was obtained from 555 mg/L MgO-NPs application with 82.50% and the lowest value was obtained from 185 mg/L MgO-NPs application with 30%.

Shoot formation rate was 60% from the control application, 42.50% from the 370 mg/L MgO application and 72.50% from the 370 mg/L MgO-NPs application. In the study, 370 mg/L MgO-NPs and 555 mg/L MgO-NPs applications increased the shoot formation rate by 20.83% and 37.50%, respectively, while all other applications had a reducing effect compared to the control. This decrease was the highest with 50% with 185 mg/L MgO-NPs administration (Table 1).

Number of Shoots:

In the study, the highest number of shoots was obtained from 61.25 to 370 mg/L MgO-NPs application, followed by 36.25 to 555 mg/L MgO-NPs application. Control and 370 mg/L MgO applications gave the same shoot number value as 21.75, while 185 mg/L MgO-NPs application gave the lowest value with 3.75. Different magnesium nanoparticles increased the shoot number compared to the control treatment. Compared to the control, this increase in the number of shoots was 181.61% higher in the 370 mg/L MgO-NPs application and 66.67% in the 555 mg/L MgO-NPs application. 185 mg/L MgO-NPs application showed a reducing effect (Table 1).

Average number of shoots per explant (ANSPE):

The mean values of the number of shoots per explant determined in the cowpea at different doses varied between 1.25-17.50. In terms of applications, the highest number of shoots per explant was obtained from the application of 370 mg/L MgO-NPs with 17.50 units, and the lowest with 1.25 units from 185 mg/L MgO-NPs application. The number of shoots per explant obtained from other applications was determined as 6.50 in the control application, 6.75 in the 370 mg/L MgO application and 10 in the 555 mg/L MgO-NPs. The decrease in the number of shoots per explant compared to the control application showed itself in the application of 185 mg/L MgO-NPs. Aasim (2010) reported that the value obtained by applying different doses of BAP and NAA to the number of shoots per explant in cowpea varies between 0.33 and 2.94, and the value obtained by the same researcher by applying different doses of TDZ varies between 1.89 and 2.78. The findings obtained from the study were higher than the results of Aasim (2010) (Table 1).

Shoot length:

In terms of shoot length, different MgO applications created significant differences. The shortest shoot was 0.18 cm in the 185 mg/L MgO-NPs application, and the longest shoot length was 2.08 cm in the 370 mg/L MgO-NPs application. 370 mg/L MgO-NPs application was followed by 555 mg/L MgO-NPs (1.45 cm) and 370 mg/L MgO (1.20 cm) applications. Except for the 185 mg/L MgO-NPs application, the other 370 mg/L MgO, 370 mg/L MgO-NPs and 555 mg/L MgO-NPs applications showed an increasing effect compared to the control.

The highest increase was obtained from the administration of 370 mg/L MgO-NPs with 336.84% (Table 1). In studies on the subject, shoot length differs in cowpea plant and Aasim (2010) reported that shoot length varies between 2.28-3.98 cm with the application of different BAP and NAA doses. Kondak (2019) determined the effects of MgO nanoparticles and MgSO_4 at different concentrations on shoot lengths in Odeska and Broken wheat varieties. The shoot lengths obtained were 15.05 and 15.48 cm, respectively.

Root formation rate:

Different Mg applications had significant effects on root formation rate. The average root formation rate of the applied magnesium nanoparticles varied between 10 and 27.5%. In terms of root formation rate, the highest value among magnesium nanoparticles was obtained from control and 370 mg/L MgO applications with 27.50%, this application was followed by 22.50% with 370 mg/L MgO-NPs application and 19.75% with 185 mg/L MgO-NPs applications. The lowest value was obtained from 10% to 555 mg/L MgO-NPs application. The highest value was recorded in the application of 555 mg/L MgO-NPs, with a 63.64% decrease in the variation of magnesium nanoparticles compared to the control (Table 1).

Number of roots per explant (NRPE)

Different MgO-NPs applications had significant effects in terms of root number per explant. While the maximum number of roots per explant determined in cowpea, where different doses of magnesium nanoparticles were applied, was obtained as 6.75 in the application of 370 mg/L MgO NPs, this application was followed by 2.75 in the control application. When the research results were examined, the applied magnesium nanoparticles at different doses caused significant reductions compared to the control, except for the application of 370 mg/L MgO-NPs. The maximum decrease in the number of roots per explant was determined as 99.96% in the application of 185 mg/L MgO-NPs. 370 mg/L MgO application had an increasing effect on the number of roots per explant, and this increase was determined to be 145.45% (Table 1).

Root length:

According to the analysis of variance results, root length was significantly affected by different MgO-NPs applications. It was determined that the root length values obtained from the cowpea with different doses of magnesium nanoparticles varied between 0.0150 and 157.50 mm. While the highest root length obtained from the applications was 157.50 mm from the 370 mg/L MgO application, this application was followed by the control application with 107.50 mm. In the 370 mg/L MgO application, there was an increase of 46.51% compared to the control. A decrease was determined in all other applications except this application (Table 1).

Nanoparticles (NP) have begun to be used extensively in plant tissue culture studies. It is known that the kind, type, concentration and size of nanoparticles (NPs) are effective in studies conducted within the scope of plant tissue culture. Positive effects of NPs on callus induction, shoot regeneration and explant growth were observed. The growth characteristics of *Tecomella undulata* (Roxb.) were evaluated by combining silver NPs (5-80 mg L⁻¹) alone or with 6-benzyl amino-purine (6 BAP) and indole acetic acid (IAA). Addition of silver NPs (10 mg L⁻¹) along with 2.5 mg L⁻¹ BAP and 0.1 mg L⁻¹ IAA in MS medium increased the number of fresh shoots per explant, the percentage of shoot-producing explant, as well as plant viability due to the effect on ethylene blocking. Concentrations of silver NPs above 60 mg L⁻¹ resulted in reduced shoot regeneration (Aghdaei et al.2012).

Genady et al. 2016, shoot, root length and fresh weight of copper sulfate (CuSO₄) NPs on hazelnut seedlings in *Verbena bipinnatif* at concentrations of 5.10 and 15 µM L⁻¹. They stated that 5 mg L⁻¹ CuSO₄ NP in MS medium increased shoot length (52% compared to control), root length (21% compared to control), and fresh weight (39% compared to control). In this experiment, phenolic contents were increased by the use of CuSO₄ NPs. Talankova-Sereda et al. 2016, in their study on *Mentha longifolia* L. with cobalt NPs (0.8 mg L⁻¹) and copper (0.5 mg L⁻¹) added to MS medium, the shoots of the plant, shoot length, rooting, and a significant difference in all evaluated parameters. increase was considered.

Effect of zinc oxide (ZnO) NPs (34 nm in size) at different concentrations (0, 0.1, 1.0, 10, 100 or 1000 mg L⁻¹) on microproagulated physiology and steviol glycoside (rebaudioside A and stevioside) production *Stevia Rebaudiana Bertoni* studied in the plant. The highest shoot formation percentage (89.6%) was obtained from 1 mg L⁻¹ ZnO NP concentration. Moreover, a significant increase of steviol glycosides (almost doubling compared to control) was observed in microproagulated shoots grown under oxidative stress of 1 mg L⁻¹ ZnO NPs according to HPLC results. However, antioxidant activities, formation of secondary metabolites, and physiological parameters showed a sudden decrease after crossing the threshold of 1 mg L⁻¹ ZnO NP concentration and decreased to a minimum at 1000 mg L⁻¹ (Javed et al. 2017).

Different concentrations of silver or gold NPs (1:2, 1:3, 2:1, and 3:1) alone or combined with naphthalene acetic acid (NAA) were evaluated for callus culture growth in *Prunella vulgaris* L. In combination with NAA (2.0 mg L⁻¹), callus proliferation (%) of silver NPs (30 µg L⁻¹), silver-gold NPs (1: 2), and silver-gold NPs (2: 1) 100) compared to control (95%) (Fazal et al. 2016).

Addition of silver NPs onto MS medium evaluated callus induction in *Solanum nigrum* L. Different concentrations of aqueous silver NPs were used in MS medium (0, 2, 4 and 8 mg L⁻¹). In the control treatment (no silver NPs), compact calli of white and greenish color were obtained after 10 days, while fragile watery calli of white, greenish or yellowish color were observed after 10-13 days on culture supplemented with silver NPs. In addition, when silver NPs were added to the medium, callus fresh weight and callus formation density increased, but deformations in callus morphology were found especially in high concentrations of NPs (Ewais et al. 2015). For callus induction from leaf explant, the effect of multi-walled carbon nanotubes (25-500 mg mL⁻¹) with B5 nutrient medium was investigated and callus growth was observed significantly at a concentration of 25 to 50 mg mL⁻¹. But at higher concentrations (100-500 mg mL⁻¹) reduced callus biomass (Ghorbanpour and Hadian 2015).

From all the studies mentioned, it is possible to conclude that NPs have positive or negative effects on seedling, callus proliferation, shoot proliferation and somatic embryogenesis, depending on the concentration used in plant tissue culture studies, but there are still many parameters that need to be investigated to elucidate the mechanism of action of Nanoparticles. As a result; It was determined that different doses of MgO-NP caused significant changes on the characters studied in the cowpea plant. It has been determined that Mg doses have doses that will encourage plant growth. In particular, it was determined that 370 mg/L MgO-NPs application gave the highest values among all parameters examined. It has been determined that MgO NP has a stimulating and regulating effect on plant growth in cowpea (Figure 2:C, D).

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Contributions

KH conceived and designed the experiments. RK and MO performed the experiments and analyzed the data. AA and HN wrote and revised the manuscript. KH and AA read and approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

A) . SEM image of MgO-NPs synthesized by Walnut Shell extract, B) XRD pattern of MgO nanoparticles C) The FT-IR spectrum of green synthesized MgO-NPs using walnut Shell extract

Figure 2

A) Cultured explants B) morphogenesis C) shoot and root formation D) Regenerated cow pea plantlets.