ENHANCEMENT OF ACTIVE COMPOUNDS PRODUCTION AND STUDYING THE GENETIC VARIATION IN CALLUS OF *OREGANO* BY ADDING NANOMATERIALS

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ABSTRACT

This study aimed to investigate the stimulation of the formation of phenolic compounds in callus cultures of *Origanum vulgare* using nanomaterials. Different combinations of plant growth regulators (NAA, BAP, and TDZ) were applied to form the calli from leaf explants. The optimal effect was achieved with a combination of 1 mg/L NAA and 0.5 mg/L BAP, which resulted in an initiation rate of 100% along with significant increase in both fresh and dry weight. The addition of silver nanoparticles (Ag-NPs) and titanium dioxide nanoparticles (TiO_2-NPs) at different concentrations (1, 2, and 3 mg/L) to the culture medium led to a substantial decrease in the fresh and dry weight of calluses with a significant increase in the content of tested phenolic compounds compared to the control. The effective treatment was 2 mg/L Ag-NPs, which enhanced the production of most phenolic compounds. Additionally, the RAPD-PCR study indicated the presence of genetic variation in the callus cultures under nanoparticle stress.

Keyword: Callus cultures, nanoparticles, phenolic compounds, RAPD-PCR.

تحسين إنتاج المركبات الفعالة ودراسة التباين الوراثي في كالس الاوريغانو بإضافة مواد نانوية

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المستخلص

هدفت هذه الدراسة إلى التحقيق في تحفيز تكوين المركبات الفينولية في مزارع الكالس من الأوريجانو باستخدام المواد النانوية. تم تطبيق مجموعات مختلفة من منظمات نمو النبات (NAA و BAP و TDZ) لتشكيل الكالس من الأوراق. تم تحقيق التأثير الأمثل باستخدام مزيج من 1 مجم/لتر NAA و 0.5 مجم/لتر BAP، حيث أدى إلى معدل نشوء للكالس بنسبة 100٪ مع زيادة كبيرة في كل من الوزن الطري والجاف. أدت إضافة جزيئات الفضة النانوية (Ag–NPs) وجسيمات ثاني أكسيد التيتانيوم النانوية (TiO_2–NPs) بتركيزات مختلفة (1، 2، 3 مجم/لتر) إلى وسط الاستزراع إلى انخفاض كبير في الوزن الطري والجاف للكالس مع زيادة معنوية في محتوى المركبات الفينولية المختبرة مقارنة مع المجموعة الضابطة. المعاملة الفعالة كانت 2 مجم/لتر Ag–NPs، والتي عززت إنتاج معظم المركبات الفينولية. بالإضافة إلى ذلك، أشارت دراسة RAPD–PCR إلى وجود تباين وراثي في مزارع الكالس تحت جهد الجسيمات النانوية.

الكلمات المفتاحية: مزارع الكالس، الجسيمات النانوية، المركبات الفينولية، RAPD–PCR.

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INTRODUCTION

Oregano (*Origanum vulgare* L.) is an important herbal and medicinal plant belonging to the Lamiaceae family, and has over a hundred phytochemicals in its various extracts (26, 33). The main types of biological components included in this plant are essential oils and phenolic compounds, which are responsible for its medicinal uses (11). The 28 ingredients of phenolic compounds were observed by Parra *et al*. (28), while 16 types of phenols were detected by Khorsand *et al*. (20). Rosmarinic acid, chlorogenic acid, quercetin, caffeic acid, gallic acid, apigenin, luteolin, kaempferol, and naringenin were the major phenolic metabolites identified in oregano plants. Most of these substances offer a wide range of therapeutic potentials, including their impact on stomachaches, rheumatoid arthritis, urinary issues, respiratory illnesses, diabetes, and cardiac diseases (33, 28). Additionally, phenolic compounds possess strong antioxidant properties that reduce many oxidative stressors (4). In general, different plant growth regulators (PGRs) are used to induce various *in vitro* plant cultures to enhance the production of many secondary metabolites (SM). The elicitation and incorporation precursor strategies significantly stimulated the content of different bioactive phytochemicals in organ and cell cultures (13). According to recent studies, the nanoparticles (NPs) applied to the plant *in vitro* growth media may function as both a source of nutrients and an abiotic elicitor (2, 6, 10, 14). Furthermore, scientific studies have shown that NP elicitation alters many physiological activities, such as reproduction, photosynthetic rate, seed germination (17, 26), and the production of many primary and SM (35). There are other responses that occur in the plant when exposed to NPs. Chenga *et al*. (7) found that the transcript of genes involved in the biosynthesis of plant hormones like ABA and IAA increased after exposure to NPs in the *Brassica napus* plant. Additionally, many therapeutic compounds have also been identified as positive effects of NPs (6). According to scientific beliefs, NPs can, like any other form of abiotic stress, generate reactive oxygen species (ROS), which then stimulate antioxidant defenses to lower ROS activities (22). Moreover, ROS is partially detoxified by phenolic substances that function as electron donors in cellular structures (36). For instance, high proliferation of callus and augmentation of phenolics, flavonoids, and phenylalanine ammonialyase (PAL) activity were observed after eliciting *Caralluma tuberculate* culture with silver nanoparticles (Ag-NPs) (5). Where titanium dioxide nanoparticles (TiO_2-NPs) increased lignans and total phenol in cell cultures of *Linum usitatissimum* (18), and improved formation of α-tocopherol in callus cultures of *Argania spinosa* (9). The antibiotic production was also increased in microorganisms after using TiO_2-NPs (37). Additionally, genetic variation and occurrence of mutations were observed by Tymoszuk and Kulus (36) in the shoot culture of *Chrysanthemum* induced by Ag-NPs using ISSR and RAPD markers. The aim of this study was to find the best method to form calluses from leaf explants and see how Ag-NPs and TiO_2-NPs affected callus growth, phenolic compound production, and genetic variation in the callus cultures that were made.

MATERIALS AND METHODS

Young and healthy leaves of the *Origanum vulgare* plant, at the flowering stage, were collected in the spring season and sterilized using sodium hypochlorite according to (1).

Establishment of callus cultures

The sterilized leaves were cultured on Murashige and Skoog (MS) medium (25), augmented with (0, 0.5, 1, and 1.5 mg/L) Naphthaleneacetic acid (NAA), either alone or combined with 0.5 mg/L Benzylaminopurine (BAP) or 0.5 mg/L Thidiazuron (TDZ). Steps of preparation and sterilization of the medium were achieved according to (1).

Effects of Ag-NPs and TiO_2-NPs on callus weights, phenolic compounds, and genetic variation: Two nanomaterials at 50 nm were used for elicitation: Ag-NPs (obtained from Nanjing Nano Technology Co., Ltd., China), and TiO_2-NPs (obtained from Hongwunanomter, China). Each type of NP was added at three concentrations (1, 2, and 3 mg/L) to the maintenance medium [MS medium had the best combination of PGRs (0.5 mg/L BAP and 1 mg/L NAA)]. All components were autoclaved for 20 min at 121˚C. the initiation weight of callus tissue

was 200 mg, which planted on each replicate of elicitation media. The cultures were kept under the darkness conditions at 25 ± 1 °C. Fresh and dry weights were recorded after 30 days (3).

Extraction of phenolic compounds and HPLC conditions: The extraction of phenolic compounds was achieved according to procedures illustrated in (20). Sex standards of phenolic compounds were obtained from Sigma-Aldrich, USA: caffeic acid, apigenin, rutin, hesperidin, rosmarinic acid, carvacrol, naringenin, and vitexin. HPLC analysis was performed using a system (Shimadzu LC-10AV, Japan), equipped with a binary delivery pump (LC-10AV) and a UV-Vis 10A-SPD spectrophotometer. Separation of phenolics was done using column C18-DB (50 x 2 mm I.D., 3 µm particle size). The mobile phase was made up of two solvents: solvent A (0.05 % trifluoroacetic acid (TFA) in de-ionized water) and solvent B (0.05 % TFA in methanol). The gradient program ran from 0% B to 100% B for 10 min. The pH was 2.5.

DNA isolation and PCR amplification

Ten primers were selected to use in the random amplified polymorphic DNA (RAPD-PCR) test; their sequences are given in **Table (1).** The samples of control and treated calluses with NPs were dried, and their DNA was extracted using a genomic DNA mini kit for plants (Geneaid, Taiwan). The verification of purified DNA was confirmed by gel electrophoresis and a Nano-Drop spectrophotometer. The PCR reaction had a total volume of 20 µl and utilized the Bioneer PCR master mix (South Korea). The amplification of PCR was performed using a thermocycler (Bio-Rad, USA). Following the initial denaturation at 95 °C for 5 min, 40 cycles were applied (denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 1 min). The final extension step was carried out at 72 °C for 10 min.

Table 1. List of RAPD primers used in polymorphism studies

No.	Primers	Sequence	%GC
1	OPA-04	5´-AATCGGGCTG-3´	60
2	OPA-06	5´-GGTCCCTGAC-3´	70
3	OPA-08	5´-GTGACGTAGG-3´	60
4	OPA-09	5´-GGGTAACGCC-3´	70
5	OPD-02	5´-GGACCCAACC-3´	70
6	OPD-06	5´-ACCTGAACGG-3´	60
7	OPD-08	5´-GTGTGCCCCA-3´	70
8	OPD-10	5´-GGTCTACACC-3´	60
9	OPD-14	5´-CTTCCCCAAG-3´	60
10	OPS-19	5´-GAGTCAGCAG-3´	60

Statistical Analysis

20 replicates were used for each treatment of callus induction, 15 replicates for each NPs elicitation treatment, while 3 replicates for HPLC analysis. ANOVA analysis of variance was performed using SPSS-23 software. Duncan's multiple range test was used to estimate the comparison between values at a 5% level (31). The molecular information was analyzed according to the presence of a band, indicated by (1), and its absence by (0). The genetic distances were assessed using PAST software version 1.94b, based on the Jaccard coefficient method.

RESULTS AND DISCUSSION

Callus induction: The results showed that all treatments were able to induce callus production from leaf explants (**Table 2)**. The combination of 1 mg/L NAA and 0.5 mg/L BAP was the most effective treatment to induce the development of callus cultures and was regarded as the maintenance medium. It resulted in significant maximum callogenesis frequency and callus weights (100%, 832.2 mg, and 82.2 mg, respectively). Most treatments induced the initiation of callus during the second week of culture, while the control treatment started the induction during the fourth week, producing significantly the lowest values of the same parameters (30%, 109.7 mg, and 10.3 mg, respectively).

Estimation of phenolic compounds in leaves of *in vivo* plant and callus cultures: The concentration of phenolic compounds in the leaves of *in vivo* plants (grown in the field) and *in vitro* cultures (callus) are compared in

Table 3 and **Figure 1**. Compounds like caffeic acid, apigenin, and rutin were found in higher amounts in the field plants than in callus cultures. While the content of hesperidin, rosmarinic acid, carvacrol, naringenin, and vitexin compounds was elevated in callus cultures compared to field plants (at a percentage increase that ranged between 20.1% to 53.6%).

Table 2. Effect of different combinations of PGRs on callus induction from the leaves of *O. vulgare*

PGRs (mg/L)			Callus induction		
NAA	BAP	TDZ	Callusing %	Fresh w. (mg)	Dry w. (mg)
0	0	0	30	109.7 g	10.3 e
0.5	0	0	95	604.7 bcd	65.9 ab
1.0	0	0	100	776.5 ab	79.9 a
1.5	0	0	95	537.2 cde	51.2 bcd
0.5	0.5	0	100	705.8 abc	77.6 a
1.0	0.5	0	100	832.2 a	82.2 a
1.5	0.5	0	95	411.6 e	46.6 cd
0.5	0	0.5	85	478 de	55.1 bc
1.0	0	0.5	80	352.9 e	37.2 d
1.5	0	0.5	80	379.6 e	42.5 cd

Means followed by the same letters in each column are not significantly different (at the 5% level) using Duncan's multiple range tests.

Table 3. Estimation of phenolic compounds in an *in vivo* plant's leaves and callus culture of *O. vulgare*

Phenolic compounds (ppm)	*In vivo* plant	Callus culture (Without elicitation)	Rate of increase or decrease
Caffeic acid	272.3 a	198.8 b	- 26.9 %
Apigenin	249.0 a	170.5 b	- 31.5 %
Rutin	202.2 a	167.8 a	- 17.0 %
Hesperidin	420.2 b	556.8 a	+ 32.5 %
Rosmarinic acid	164.5 a	197.7 a	+ 20.1 %
Carvacrol	105.2 b	146.1 a	+ 38.8 %
Naringenin	124.0 b	190.5 a	+ 53.6 %
Vitexin	530.7 b	679.8 a	+ 28.0 %

Means followed by the same letters in each column are not significantly different (at the 5% level) using Duncan's multiple range tests.

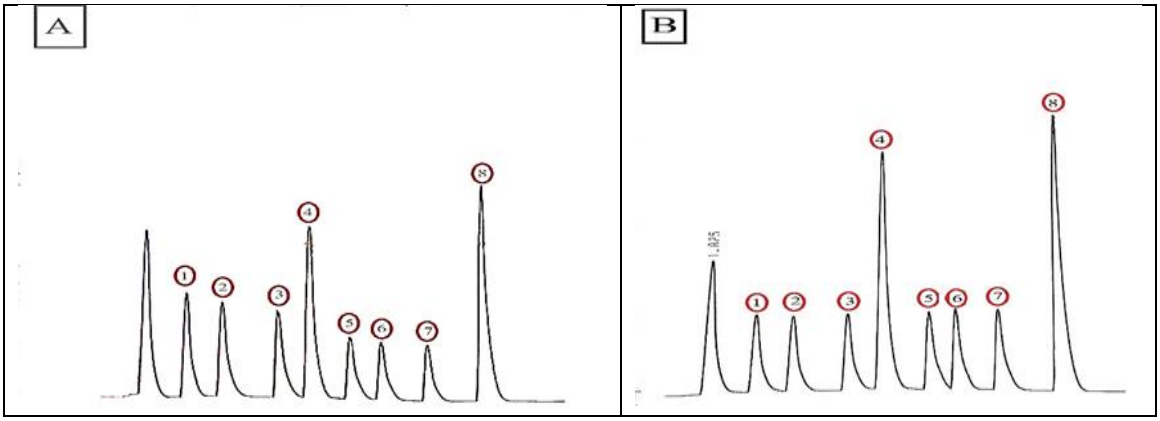


Figure 1. HPLC profile of phenolic compounds in *O. vulgare*: (1. caffeic acid, 2. apigenin, 3. rutin, 4. hesperidin, 5. rosmarinic acid, 6. carvacrol, 7. naringenin, and 8. vitexin); (A) *in vivo* plant; (B) callus culture of control treatment.

Effect of nanoparticles on callus generation and phenolic accumulation: The callus tissues were replanted on a maintenance medium (MS medium having best

combination of PGRs (1 mg/L NAA and 0.5 mg/L BAP) for callus induction). The fresh and dry weights were recorded at the end of the elicitation time (30 days). As shown in Table 4 and Figure 2, all concentrations of Ag-NPs and TiO_2-NPs exhibited a significant reduction in callus weights compared to the control treatment. All concentrations of TiO_2-NPs recorded the lowest weight values. The results in Table 5 indicate that using NPs individually was highly effective. Treatment with 1 mg/L Ag-NPs noticeably increased the concentration of naringenin (311.2 ppm) compared to the control and other treatments. Additionally, treatments supplemented with 2 mg/L Ag-NPs showed a significant increase in various phenolic compounds, including caffeic acid, rosmarinic acid, carvacrol, and vitexin (341.1, 444.0, 193.8, and 913.9 ppm, respectively). Furthermore, treatment with 3 mg/L Ag-NPs resulted in a significant increase in apigenin content (281.1 ppm), whereas treatment with TiO_2-NPs at 2 and 3 mg/L significantly increased the concentrations of rutin and hesperidin (by 234.0 and 681.6 ppm, respectively) compared to the control and other treatments.

Table 4. The effect of Ag-NPs and TiO_2-NPs on fresh and dry weights (mg) of callus cultures after 30 days of elicitation (the initial callus weight was 200 mg).

Weight of callus (mg)	Control	Ag-NPs			TiO_2-NPs		
	0 mg/L	1 mg/L	2 mg/L	3 mg/L	1 mg/L	2 mg/L	3 mg/L
Fresh W.	1139.3 a	928.8 b	912.2 b	923.5 b	779.5 c	788.7 c	750.8 c
Dry W.	105.1 a	87.5 b	82.3 b	84.4 b	75.8 b	78.9 b	75.7 b

Means followed by the same letters in each column are not significantly different (at the 5% level) using Duncan's multiple range tests.

Table 5. The effect of Ag-NPs and TiO_2-NPs on the accumulation of some phenolic compounds in the callus culture of *O. vulgare* after 30 days of elicitation

Phenolic compounds (PPM)	Nano elicitation						
	Control 0 mg/L	Ag-NPs 1 mg/L	Ag-NPs 2 mg/L	Ag-NPs 3 mg/L	TiO_2-NPs 1 mg/L	TiO_2-NPs 2 mg/L	TiO_2-NPs 3 mg/L
Caffeic acid	198.8 b	201.9 b	341.1 a	326.1 a	186.8 b	297.0 a	280.4 a
Apigenin	170.5 b	166.5 b	245.2 a	281.1 a	183.3 b	188.8 b	260.1 a
Rutin	167.8 bc	149.1 c	178.6 abc	212.0 ab	158.5 bc	234.0 a	230.0 a
Hesperidin	556.8 b	577.9 b	659.2 a	665.0 a	465.8 c	530.3 b	681.6 a
Rosmarinic acid	197.7 d	247.6 cd	444.0 a	343.2 b	247.3 cd	278.2 bc	284.2 bc
Carvacrol	146.1 bc	183.9 ab	193.8 a	135.6 c	117.5 c	149.7 abc	181.7 ab
Naringenin	190.5 c	311.2 a	234.1 bc	286.2 ab	208.5 c	233.1 bc	280.9 ab
Vitexin	679.8 cd	761.0 b	913.9 a	772.5 b	615.4 d	685.7 c	812.9 b

Means followed by same letters in each column are not significantly different (at 5% level) using Duncan's multiple range tests.

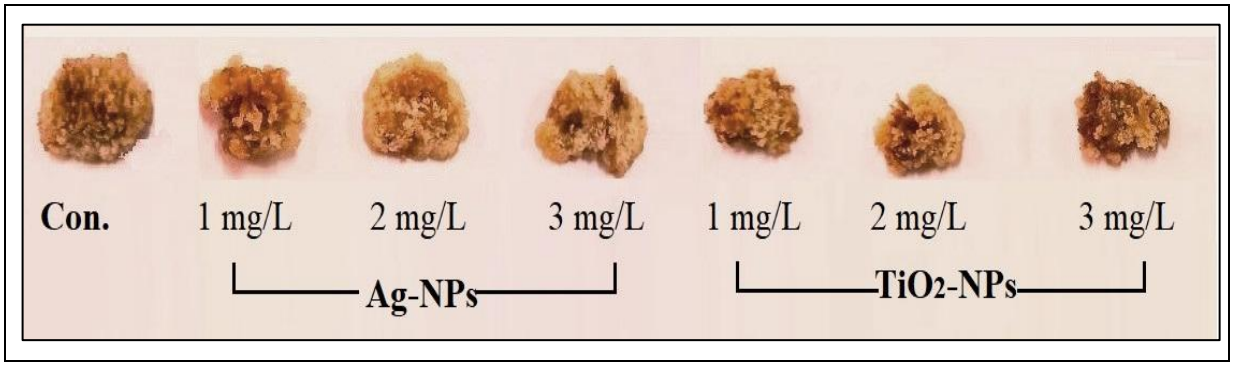
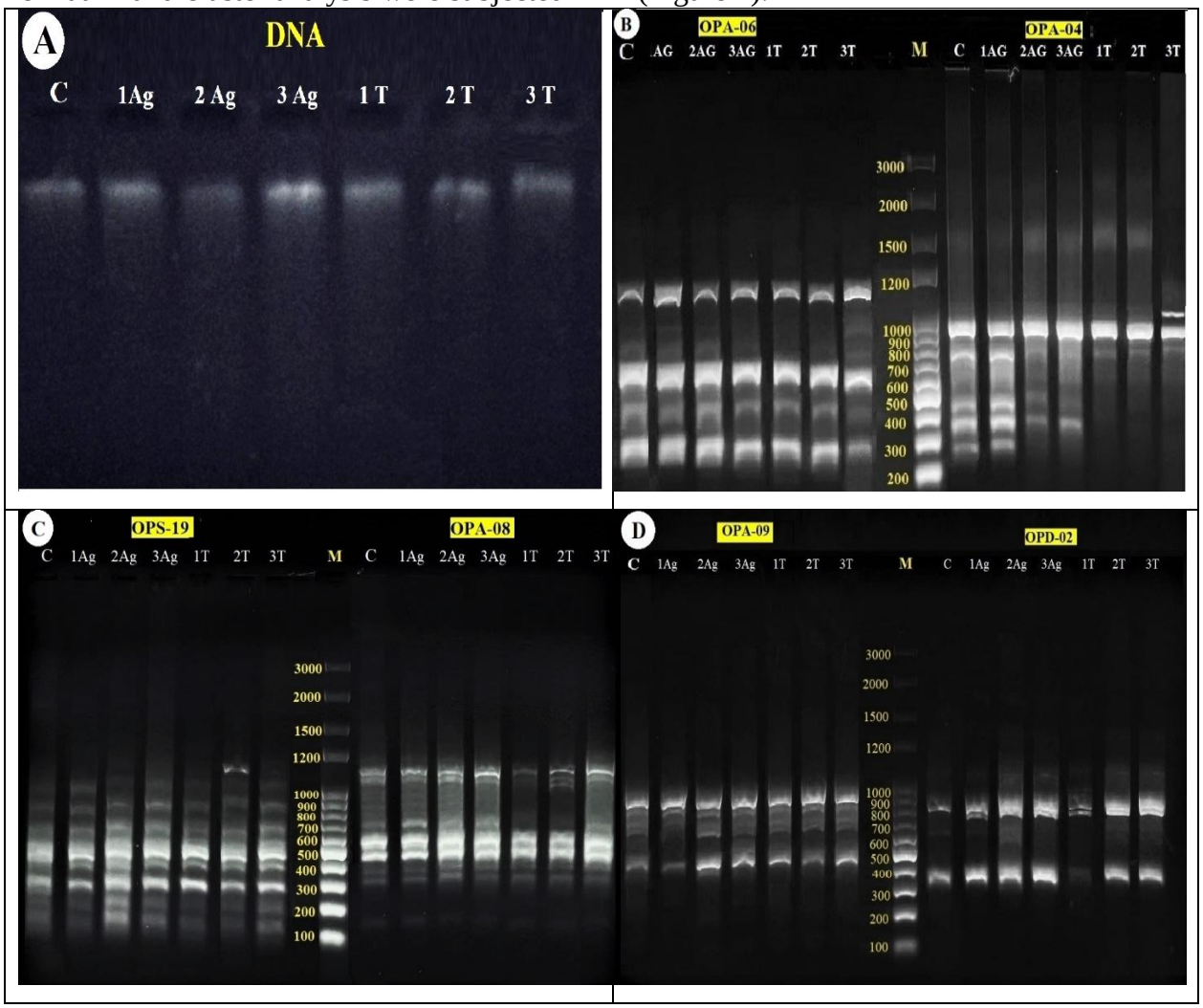


Figure 2. Morphology of callus culture of *O. vulgare* after elicitation with different concentrations of Ag-NPs and TiO_2-NPs.

Effect of nanoparticles on the genetic similarity of callus cultures (RAPD-PCR): The quality of isolated DNA was shown in Figure 3A. The isolation technique presented a concentration of DNA that varied from 78 to 125 ng/µl, with a purity range of (1.7-1.9). The amplification of RAPD primers using different

samples of DNA template was analyzed and shown in Table 6. Ten primers in total were examined, and seven of them displayed comparable banding patterns. These ten primers generated 50 bands, with sizes ranging from 150 to 1200 bp (Figure 3, B-F), 12 of which were polymorphic. The proportion of polymorphism (P%) varied from 12.5% in OPS-19 to 81.866.62% in OPA-04, with an average of 25.9% polymorphism per primer. The matrix and cluster analysis were subjected to Jaccard's similarity coefficient. The highest genetic similarity was found (0.97674) among 2Ag and 3Ag samples (these samples represent 2, and 3 mg/L Ag-NPs), while the lowest value was found between the 2Ag and 2T samples (0.8125) (Table 7). Furthermore, the cluster analysis shows that samples were grouped into two main clusters. The first group consists of control and Ag-NPs treatments, while the second group has only TiO_2-NPs treatments (Figure 4).



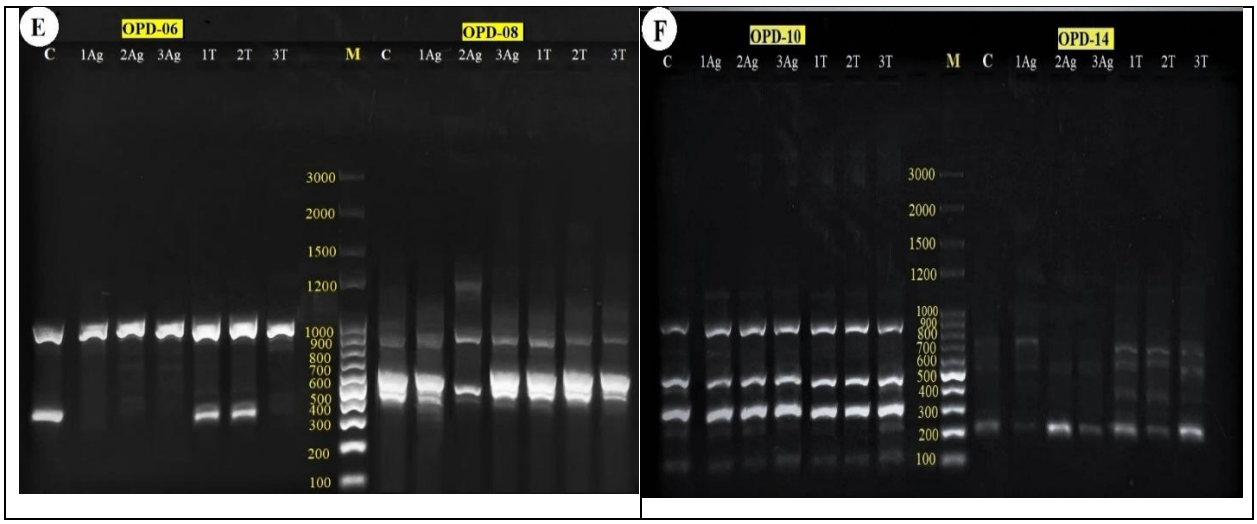


Figure 3. DNA Quality extracted from seven callus cultures of *O. vulgare* plant (A), and RAPD profile of different primers, OPA-06, and OPA-04 (B); OPS-19, and OPA -08 (C); OPA-09, and OPD-02 (D); OPD-06, and OPD-08 (E); OPD-10, and OPD-14 (F), (the marker DNA ladder is100 bp).

Table 6. Primer sequence, size range (SR), total bands (TB), monomorphic bands (MB), polymorphic bands (MP), and polymorphic percentage (P%) for different RAPD primers

NO.	Primers	Sequence (5'-3')	SR (BP)	TB	MB	PB	P%
1	OPA-04	AATCGGGCTG	300-1050	6	2	4	66.6
2	OPA-06	GGTCCCTGAC	300-1170	4	4	0	0.0
3	OPA-08	GTGACGTAGG	150-320	9	7	2	22.2
4	OPA-09	GGGTAACGCC	500-950	4	4	0	0.0
5	OPD-02	GGACCCAACC	350-850	3	2	1	33.3
6	OPD-06	ACCTGAACGG	350-1000	2	1	1	50.0
7	OPD-08	GTGTGCCCCA	500-1200	4	3	1	25.0
8	OPD-10	GGTCTACACC	100-900	6	6	0	0
9	OPD-14	CTTCCCCAAG	210-750	4	2	2	50.0
10	OPS-19	GAGTCAGCAG	150-1180	8	7	1	12.5
		Total bands		50	38	12	
		Average		5	3.8	1.2	25.9

Table 7. The similarity matrix of *O. vulgare* callus samples at various concentrations of nanoparticles based on RAPD primers

Samples	C	1 AG	2 AG	3 AG	1 T	2 T	3 T
C	1						
1 AG	0.95556	1					
2 AG	0.8913	0.93333	1				
3 AG	0.91111	0.95455	0.97674	1			
1 T	0.86957	0.86957	0.84783	0.86667	1		
2 T	0.83333	0.83333	0.8125	0.82979	0.95455	1	
3 T	0.85106	0.8913	0.86957	0.88889	0.93182	0.8913	1

C=Control; 1Ag, 2Ag, 3Ag = 1, 2, 3 mg/L Ag-NPs, respectively; 1T, 2T, 3T =1, 2, 3 mg/L TiO_2-NPs, respectively.

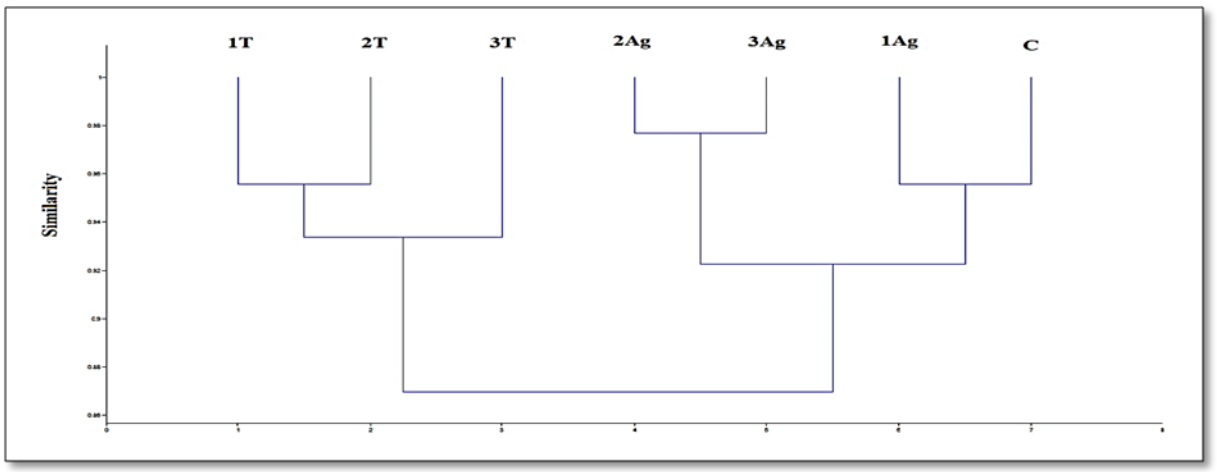


Figure 4. Cluster analysis of *O. vulgare* callus cultures treated with different concentrations of Ag-NPs and TiO_2-NPs using the Jaccard coefficient of similarity

Modern plant biotechnologies provided many proofs that callus cultures provide raw materials for plant study and productivity (9). The relationship between the level of endogenous hormones and the concentrations and quality of added growth regulators often affects the size, mass, and quality of callus formed on the culture media (16), which explains (in the current study) why the callus formed in low rate and weight in the control treatment (without PGRs). In general, auxin and cytokinin work synergistically to control cell division in plant cultures (12). Additionally, the exogenous requirements of PGRs for callus induction usually differ depending on the kind of explant, for instance, in tobacco cultures, calluses originating from leaves require exogenous cytokinins whereas those derived from stem explants do not (34). Furthermore, the callus mass is often balanced by the auxin to cytokinin ratio (8); this was seen in the callus of *O. vulgare*, where callogenesis was effectively induced by the addition of Kin to 2,4-D and BAP to NAA (21). Auxin is frequently employed at higher concentrations than cytokinin to achieve favorable outcomes in terms of callus production and percentage in plant tissue cultures (30). With regard to the content of phenolic substances in oregano plant, the HPLC analysis demonstrated that the level of these compounds could be classified into two distinct categories. The first category exhibited higher concentrations of phenolic compounds in field leaves, whereas the second category displayed higher concentrations in callus cultures. This observation agreed with findings from prior studies suggesting that the biosynthesis of some active compounds is probably related to plant organ or cell development (19) or correlated with certain ecological conditions (29), or type cultures (30). When nanoparticles are employed *in vitro*, their characteristics affect how plants react to these stimuli; some of these characteristics are surface area, particle size, crystal structure, physiochemical traits, and concentrations (32). NPs can be efficiently absorbed by plants, which then utilize them as nutrients or as elicitors to alter their cellular development or metabolic processes (9). In the current study, the significant decrease in callus biomass and increase in phenolic compound concentrations at the different NPs treatments provide the best evidence that these treatments were powerful stimulants that sped up secondary metabolic events, both at the genetic variation and phytochemical production levels. It is well known that the application of abiotic or biotic elicitors in the growth medium can increase the production of bioactive substances while increasing the activity of the defense system in response to the therapies (27). Nano-toxicity and its mechanisms of action in plants are still not well understood. However, it is hypothesized that oxidative stress brought on by ROS makes plants more susceptible to NPs-induced damage. Free radicals frequently attack cells, and when they cause harm, phenolic compounds can build up in the injured tissues and cells, which can serve as "electron donors" to support detoxification mechanisms (22). In comparison to the control, DNA

polymorphism was found in samples of callus treated with Ag-NPs and TiO_2-NPs. This result indicates the possibility of NPs to causing genetic variations. The findings of this research align with the perspective of Mehrian and Lima (23), who indicated that the use of NPs could potentially result in abnormalities in spindle fibers, loss of genetic material, and hindered DNA synthesis during the S-phase. As a consequence, these events could give rise to genetic alterations. Furthermore, Miryeganeh and Saze (24) suggested that Ag-NPs may cause epigenetic action by changing DNA methylation and/or acetylation. All these reactions can vigorously enhance the biosynthesis of various SM like phenolic compounds, alkaloids, and other phytochemicals (15). The conducted experiment revealed that the combination of NAA and BAP effectively improved the formation of callus cultures derived from *O. vulgare* leaf explants. The resultant culture exhibited a favorable response toward NPs, resulting in a marked escalation in the yield of medicinally potent and efficacious plant compounds. However, these NP treatments also induced genetic modifications, which could be advantageous or disadvantageous in the callus cultures of rosemary plants.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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