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Research Paper

Impact of Al_2O_3 NPs on Callus Induction, Pigment Content, Cell Damage and Enzyme Activities in *Ocimum basilicum* Linn.

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Abstract: With the advancement of nanotechnology, various potential applications of nanoparticles (NPs) have attracted considerable attention. In recent years, plant tissue culture applications in agricultural nanotechnology have become more popular. However, there are very few studies evaluating the effect of aluminum oxide nanoparticles (Al_2O_3 NPs) on enzyme activity and pigment content after plant tissue culture application. For this purpose, *Ocimum basilicum* L. callus growth effects, lipid peroxidation, hydrogen peroxide (H_2O_2) scavenging activity and chlorophyll content were investigated. As a result of this application, callus formation percentage and callus weight of the stem segment were found to be better than the leaf as the source of explants. The highest callus formation percentage (100%) was recorded as 741 mg stem and 324 mg leaf in (MS + 75 mg/l Al_2O_3) nutrient medium. In the (B5 + 75 mg/l Al_2O_3) nutrient medium, the stem was found to be 675 mg and the leaf 350 mg. Stress caused by Al_2O_3NP application was evaluated by chlorophyll and carotenoid pigment measurement. The highest Chl-a was detected at 75 mg / l Al_2O_3 NP concentration. The lowest total carotenoid was reported at 100 mg/l. The lowest Chl-a was detected at 25 mg / l. It was observed that the test groups treated with Al_2O_3 nanoparticle were significantly higher than the control group. In particular, the malondialdehit (MDA) level at 50 mg/l was quite high (7,409 times compared to control).

Keywords: *Al_2O_3, nanoparticles, plant tissue culture, MDA, chlorophyll, pigment*

Introduction

Nanotechnology is one of the fastest growing fields of advanced technology and therefore a source of hope for many branches of modern industry as well as medicine and pharmacy. In recent years, the use of nanoparticles (NP) in commercial products and industrial applications has increased significantly. However, the molecular level interaction mechanisms between NPs and biological systems have not been fully elucidated (Barrena *et al*., 2009; Khot *et al*., 2012). Moreover, certain NP properties, such as large specific surface area and greater reactivity, have raised problems due to their negative impact on human and environmental health (Andre and Mädler, 2006, Maynard *et al*., 2007). However, information on the fate of NPs in water and soil remains limited. With current research, positive and negative effects of NPs on plants have been reported, and researchers have investigated the effects of NPs on plant germination and growth (Khot *et al*., 2012). Also, some reports have confirmed that NPs can induce phytotoxicity and have a negative effect on seed germination and growth. However, specific properties of NPs such as size, shape and load can also be used to improve seed germination and crop performance (Khot et al., 2012). In a study focusing on NPs in edible plants, all plants exposed to or treated with NPs were reported to indicate toxicity.

Aluminum is one of the most abundant metallic elements in the world. It offers wider usage possibilities with its availability in many alloys (Johnson and Sanders, 2012; Ruan & Schuh, 2012). Aluminum oxide (Al_2O_3) is used in the health and cosmetics industry due to its high mechanical strength, hardness, wear resistance, high biocompatible structure and chemical inert properties (Lukin et al., 2001). Aluminum oxide nanoparticles are among the most preferred nanoparticles. As the element aluminum has been studied for many years, its toxicity is relatively well known. However, the toxicity of Al_2O_3 nanoparticles is still not fully known (Dağlıoğlu & Öztürk 2016; Dağlıoğlu & Öztürk 2018 a,b; Dağlıoğlu & Öztürk 2021). Possible mechanisms for the cytotoxicity of aluminum oxide NPs are still

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being debated. However, oxidative stress and DNA damage may be responsible for its cytotoxic effects (Kim et al., 2009). Sweet basil *(O. basilicum* L), a species of Lamiaceae family, is an extremely valuable plant and is grown commercially in many countries of the world (Hussain et al., 2008). The flowers and leaves of the plant are used in the food industry, either fresh or dried (Makri & Kintzios, 2007). *O. basilicum* is an anesthetic, anti-inflammatory analgesic, anti-ulcerogenic, heart stimulant, anti-tuberculosis (Limma-Netto et al., 2017; Bilal et al., 2012; Siddiqui et al., 2012). Its antioxidant, antimicrobial and antifungal potentials are high (Snoussi et al., 2016; Piras et al., 2018). In addition, it is used by the public for carminative, galactagogue, digestive system and spasm (Marwat *et al.,* 2011).

In this study, Al_2O_3 NPs and *O. basilicum* species were selected due to the above-mentioned properties. The most effective concentration was determined by applying Al_2O_3 NPs prepared in different concentrations to the plant cell culture of *O. basilicum*. After cell culture studies, Al_2O_3 NP treated *O. basilicum*. was collected from plant cell cultures and chlorophyll analysis, lipid peroxidation and hydrogen peroxide level were determined.

Materials and Methods

Plant and nanoparticle materials

Seeds of sweet basil (*Ocimum basilicum* L.) was used as plant material in this study. *O. basilicum* seeds were obtained from the Department of Field Crops, Faculty of Agriculture of Ordu University, Ordu, Turkey. Aluminum oxide nanoparticles (Al_2O_3 NPs), hydrophilic with a purity of 99% and an average particle size of 20 nm, used in toxicity tests were provided from a Nanography company from Ankara, Turkey.

Preparation of Al_2O_3 NPs test solution

To prepare stock solutions at concentrations of 0, 25, 50, 75 and 100 mg/L, the test substance was prepared in a deionized water by means of the Al_2O_3 NPs dispersion medium (Dağlıoğlu and Öztürk 2018; Özturk and Dağlıoğlu 2018). The solution was then vortexed for 20 s. Ultrasonic water bath was used to prepare stock solutions of nanoparticles.

Seed germination

Sterilization process and germination according to Açıkgöz (2021). Briefly, seeds were sterilized for 30 min in 1.95% sodium hypochlorite solution and after rinsing with sterile distilled water. The seeds were then inoculated on Murashige and Skoog (MS) basal medium; supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, 1.0 mg/L GA_3, the pH was adjusted to 5.6–5.8. Later, magenta plates were kept in 25 ± 2 °C for 16/8 h light/dark cycle in the climate chamber.

Callus formation from stem and leaf explants in media (MS and B5)

Sterile seedlings (20−22 cm long, 3 months later incubation) cut leaf and stem were used as explant source for the formation of callus cultures. Stem and leaf explants were used in the study and different concentrations of Al_2O_3 (0.0, 25, 50, 75 and 100 mg/l) NPs were added to MS and B5 growth medium with 2.5 mg/L NAA (naphthalene-acetic acid)+0.5 mg/L KIN (kinetin), 3% sucrose and 0.8 % agar, kept in climate room at 25±2 °C for 16/8 h light/dark conditions. The calluses were taken to subcultures twice with four weeks interval (using the same hormone combination and growth medium).

Nanoparticle characterization

The crystal structure of the Al_2O_3 NPs was characterized by X-ray diffraction (XRD) (Rigaku Smartlab model). The diffraction patterns were recorded at 2-Theta angular range of 20^0-90^0, 40 kV and 30 mA. The diffraction patterns of Al_2O_3 powders were compared with ICDD (PDF-4 + 2015 RDB) database. Al_2O_3 NPs size and distribution were performed by scanning electron microscopy (SEM, Hitachi, SU 1510) (Fig. 1).

Preparation of Basil extract

For water and ethanol extract, 25 g of the dried basil leaves was made into a fine powder in porcelain mortar and mixed with 500 ml of boiling water with a magnetic stirrer for 15 min. The residue was re-extracted until the extract solvents became colorless. The resulting extracts were obtained by Whatman. No. 1 was filtered and collected. The ratio of leaf powder and solvent is 1: 5 (W /v/g/ml). The final

mixture, Whatman No. 4 filtered under suction with 4 filter paper. The very fine particles remaining in the filtrate were separated by centrifugation using centrifugation at 7000 rpm (Mata *et al*., 2007).

Lipid peroxidation

Lipid peroxidation determines the amount of malondialdehyde (MDA) that causes cell membrane damage. MDA was determined according to the method described by Lutts et al., 1996. Briefly, 200 mg of the plant was weighed and 5 ml of 0.1% trichloroacetic acid (TCA) was added thereto, which was centrifuged at 12500 rpm for 20 minutes. 3 ml of supernatant was taken from the 5 ml extract. On this supernatant, 3 ml 0.1 %TCA in 20 % thiobarbuturic acid (TBA) was added. The mixture was kept in a hot water bath at 95 °C for 30 minutes. Then, the absorbance values of A532 and A600 nm were read in the spectrophotometer. MDA concentration was determined as µmol/g T.A using ı "extinction coefficient" of 155 mM^{-1} cm^{-1} (Sevengör et al., 2011).

H_2O_2 scavenging activity

The ability to remove hydrogen peroxide of ethanol basil extract was determined according to the method of Ruch et al., (1989). H_2O_2 solution (40 mM) in phosphate buffer (pH 7.4) was prepared. Extracts in distilled water (50 µg/mL) were added to H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution is phosphate buffer without H_2O_2. The ethanol basil extract and the H_2O_2 cleaning percentage of standard compounds were calculated as follows (Gülçin et al., 2003; Gülçin and Aboul-Enein 2007):

$$H_2O_2 \text{ scavenging activity} = \left(\frac{Acontrol - Asample}{Acontrol}\right) x100$$

Chlorophyll analysis

At the end of the exposure period, 80% acetone solution and quartz sand were added to crushed Al_2O_3 NPs applied to fresh chlorophyll analysis. For the chlorophyll analysis, 80% acetone solution and quartz sand were added to the Al_2O_3 NPs applied basil plant and crushed. The resulting extract was taken to the centrifuge tube and 4 ml of 80% acetone was added. After centrifugation, the Whatman black band was filtered through filter paper, and the final volume was completed to 10 ml. The obtained liquid was read on the spectrophotometer at 450, 645 and 663 nm. To prevent experimental errors, three groups were studied for each sample. The amounts of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid were calculated using the following formulas.

The amounts of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid using the following formulas (Odabaş, 1981).

Chlorophyll a (mg/g texture weight) = [12.7x(D663)-2.69x(D645)] * (V/1000.A)
Chlorophyll b (mg/g texture weight) = [22.9x(D645)-4.68x(D663)] *(V/1000.A)
Total chlorophyll (mg/g texture weight) = [20.2xD645) +8.02x(D663)] *(V/1000.A)
Carotenoid (mg/g texture weight) = 4.07x D450- [0.0435x Chlorophyll a + 0.3367x Chlorophyll b]
D: wavelength, V: 100% acetone final volume, A: weight of leaf tissue in grams.

Data Analysis and statistical evaluation

Significant differences between the control and the test groups were determined by multiple comparison of one-way analysis of variance (ANOVA) and Tukey test. The *P* values show significant differences of 0.05 ($P \leq 0.05$).

Results

Callus formation from stem and leaf explants in media (MS and B5),

As a result of the examinations performed after the first planting medium and subcultures, the percentage of callus formation and callus weight were found to be better than the leaf in hormone combinations where the stem segment was used as the source of explants. Callus formation percentage, callus weight and callus scoring are given in Table 1 and 2. The highest callus formation percentage (100%) and callus weight 741 mg and 675 mg were obtained from the two main nutrient media (MS+75 mg/l Al_2O_3 NPs and B5+75 mg/l Al_2O_3 NPs).

Table 1. The effect of Al_2O_3 NPs concentrations applied to leaf and stem explants on callus formation percentage (%) and callus weight (mg) (MS media)

	Leaf			**Stem**		
Al_2O_3 NPs 0 (control)	Percentage of explants forming callus (%)	Callus weight (mg)	Leaf Score (0-4)	Percentage of explants forming callus (%)	Callus weight (mg)	Stem Score (0-4)
	50	160	2	100	100	2
	60	90	1	100	379	3
	90	300	2	100	173	2
	90	47	1	100	325	3
	90	85	1	100	190	2
25 mg/l	90	260	2	100	574	3
	90	247	2	90	557	3
	80	220	2	100	362	2
	70	217	2	70	500	3
	80	209	2	80	554	3
50 mg/l	50	103	2	60	225	2
	40	70	1	50	72	2
	40	80	1	70	190	2
	60	200	2	60	204	2
	40	79	1	80	327	2
75 mg/l	60	40	1	90	732	3
	100	312	2	100	653	3
	100	**324**	**2**	**100**	**741**	**3**
	80	148	1	80	300	2
	80	347	2	80	276	2
100 mg/l	70	100	1	30	270	2
	100	379	2	100	245	3
	80	173	1	80	368	2
	90	325	2	90	621	3
	60	190	2	80	273	2

Footnote: Scoring is calculated over 4 points. Here, criteria such as callus formation percentage, callus formation direction, callus type and weight were taken into account. However, no information is given about the direction and type of callus formation in the Table 1. Samples meeting all of these criteria received 4 points, while those meeting only one received 1 point.

Considering the effect of Al_2O_3 NPs on the percentage (%) of explants that form callus in the MS media, 50 mg/l Al_2O_3 NPs for leaf and stem are quite low. Again, it is this concentration that has the least effect on callus weight.

In the B5 nutrient medium as seen in the above table, the percentage of the explants that produced the lowest callus for the leaf and stem and the callus weight were recorded at 100 mg/l Al_2O_3 NPs concentration.

Between the two main nutrient media (MS and B5) used in the study, the highest percentage of callus formation (100%) and callus weight were obtained from the application of 324, 741 mg and 350, 1600 mg and 75 mg/l Al2O3 NPs, respectively. When leaf and stem explants, which are callus sources, were compared themselves, it was determined that stem explants stood out with 741 and 675 mg in terms of callus weight. Many studies on the subject have shown that the medium used, hormone combination and explant source are effective in callus formation.

Table 2. Callus formation, callus weight and scoring in leaf and stem explants (B5 media)

	Leaf			Stem		
Al_2O_3 NPs	Percentage of explants forming callus (%)	Callus weight (mg)	Leaf Score (0-4)	Percentage of explants forming callus (%)	Callus weight (mg)	Stem Score (0-4)
0 (control)	20	47	1	30	100	2
	80	132	2	80	304	2
	50	047	1	50	140	2
	50	142	2	50	142	2
	10	28	1	10	54	2
25 mg/l	60	140	1	60	240	2
	100	200	2	100	500	3
	100	324	2	100	514	3
	80	156	1	80	356	2
	80	141	2	80	248	2
50 mg/l	40	127	1	50	224	2
	30	132	2	50	270	2
	40	100	1	40	175	2
	70	196	2	70	270	2
	60	128	2	60	255	2
75 mg/l	100	230	2	100	530	3
	90	198	2	90	450	3
	100	**350**	**2**	**100**	**675**	**4**
	70	356	3	70	500	3
	80	220	2	80	440	3
100 mg/l	10	50	2	10	75	2
	10	14	1	10	80	2
	10	17	1	10	64	2
	60	175	2	60	210	2
	0	0	0	40	140	2

Footnote: Scoring is calculated over 4 points. Here, criteria such as callus formation percentage, callus formation direction, callus type and weight were taken into account. However, no information is given about the direction and type of callus formation in the Table 2. Samples meeting all of these criteria received 4 points, while those meeting only one received 1 point.

Physicochemical characterization of Al_2O_3 NPs

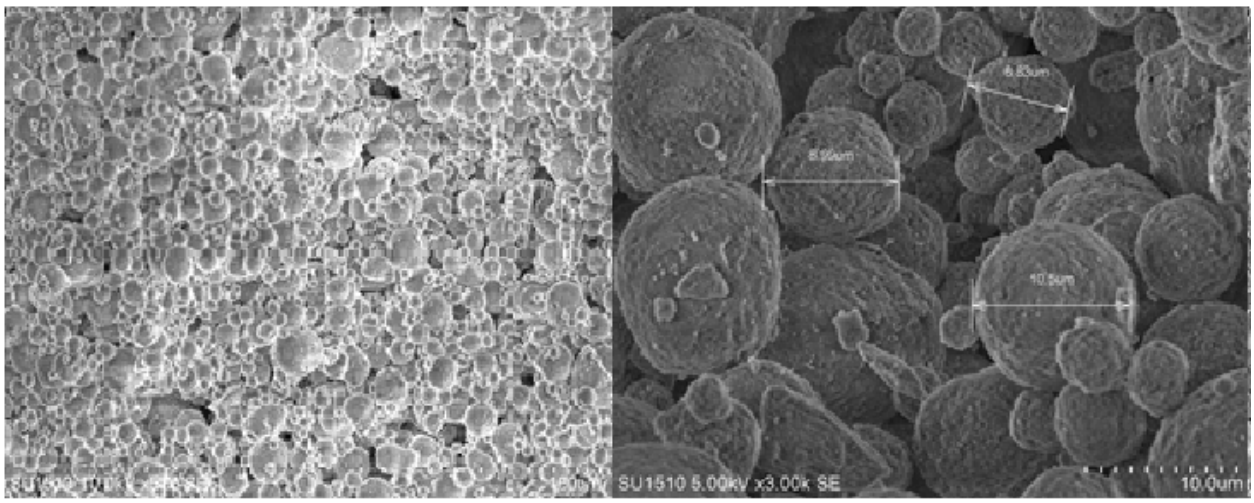

Figure 1. SEM image of the Al_2O_3 NPs

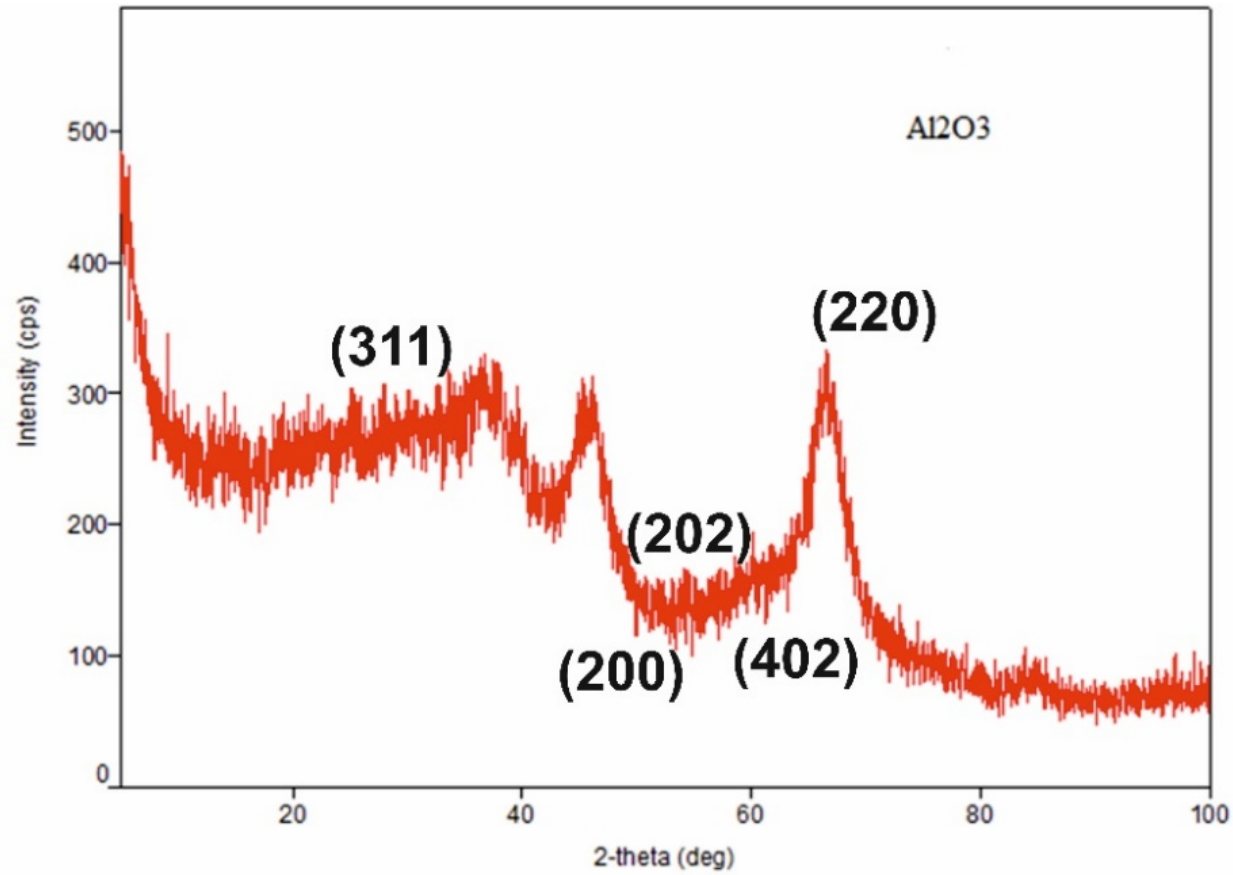

Figure 2. X-ray diffraction (XRD) analysis of powdered Al_2O_3 NPs.
Compared to standard PDF cards, the samples analyzed were found to be compatible with the Al_2O_3 element. However, some peaks did not match Al_2O_3. This is because the particles are 95% pure. This is because the particles are 95% pure.

Pigment analysis

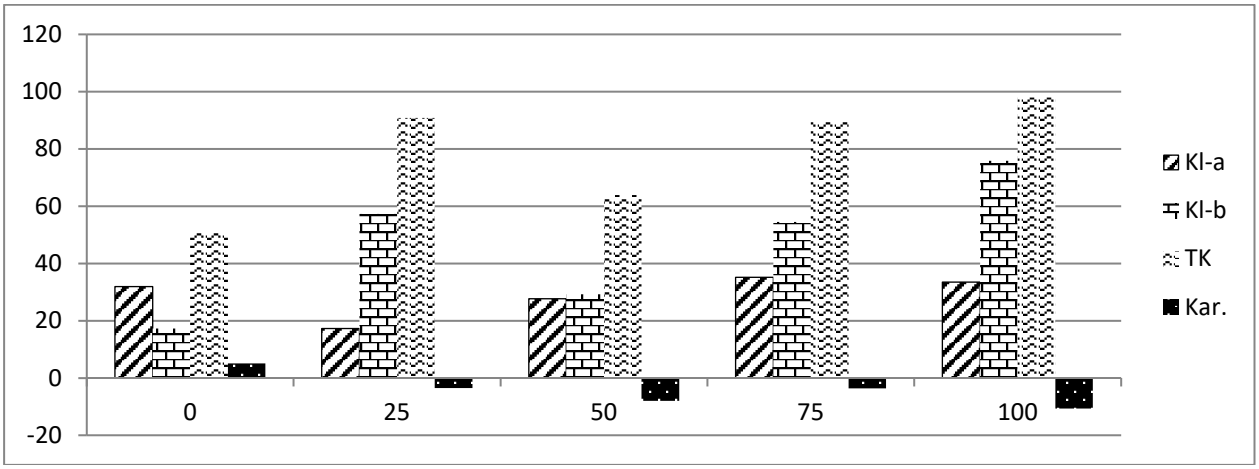

Figure 3. Pigment contents in *O. basilicum* exposed to Al_2O_3 NPs.

Considering that photosynthesis plays an important role in photosynthesis II (PSII) in response to environmental stress, analysis of chlorophyll fluorescence and evaluation of CO_2 assimilation in the administration of Al_2O_3 NPs may reflect PSII behavior (Biswal and Biswal 1999). The chlorophyll content of the extracts obtained from the *O. basilicum* leaves exposed to Al_2O_3 NPs at concentrations of 0, 25, 50, 75 and 100 mg/L was quite different. Chlorophyll a content was found to be high in low Al_2O_3 NPs concentrations compared to the control group. It was found to be low in groups exposed to high

Al_2O_3 NPs concentrations. According to the control group, it was noted that it decreased by 25, 50 mg / L, 61.7% and 13.5% and increased by 10% and 4% at 75 and 100 mg / L, respectively.

Table 3. H_2O_2 and CAT activities with MDA level in *O. basilicum* treated with AL_2O_3 NPs

Samples	MDA	H_2O_2
0 mg/L	1.158	4.00
25 mg/L	6.70*	4.00
50 mg/L	8.58*	3.972*
75 mg/L	1.212	4.00
100 mg/L	1.806	4.00

Lipid peroxidation, which was measured as the formation of MDA production, was observed to be quite high in the test groups treated with Al_2O_3 nanoparticle compared to the control group. Especially, the MDA level at 50 mg/L was quite high (7.409 times compared to the control). Lipid peroxidation at a concentration of 25 mg/L was higher than the control group (P <0.05). Lipid peroxidation at the highest dose (100 mg/L) was higher than the control (1.55 times), but reduced by 0.74 times (P *<0.05*) compared to 25 and 50 mg/L concentrations.

Discussion

Many studies on the subject have shown that the medium used, the combination of hormones and explants are effective in the formation of callus (Hariprasath et al., 2015; Dhas et al., 2016; Jin et al., 2017; Krishnan and Siril, 2017; Açıkgöz, 2020; Açıkgöz, 2021). Optimum callus formation from explant sources *in vitro* can be achieved by selecting the most appropriate source of explants (root, stem, leaf, hypocotyl and epicotylone) according to the species, using the appropriate nutrient medium (MS, B5, SH, LS and NN) and finally the correct hormone or it is provided with hormone (cytokinin + auxin) combinations. Many studies have been carried out to induce callus formation in many species. In most of these studies, stem explants induced callus formation more than leaves, while leaf explants induced more callus formation in some (Zinhari et al., 2016; Hosseini et al., 2017; Açıkgöz et al., 2018; Açıkgöz et al., 2019). It has been reported by many researchers that elicitor treatments are very effective in cell growth and callus formation. The age of cell culture (Namdeo, 2007; Kang et al., 2009), the duration of exposure to elicitors and the type of elicitor play important roles in increasing the effectiveness of these treatments (Kubeš et al., 2014). In previous studies, some researchers reported that some compounds such as CdCl2, AgNO3 inhibit cell growth and callus formation (Zhao et al., 2010; Sivanandhan et al., 2014; Zaker et al., 2015; Gonçalves et al., 2019). However, some researchers reported that some elicitors such as AgNO3 and its derivatives support cell growth and callus formation at appropriate concentrations. (Yan et al., 2006; Deepthi and Satheeshkumar, 2016; Singh et al., 2017; Roy and Bharadvaja, 2019).

In this study, the toxicological profile and general mechanisms related to the chronic toxicity of Al_2O_3 NPs applied to *O. basilicum* cell culture were investigated. Preliminary characterization of Al_2O_3 NPs was performed by SEM and XRD analyzes before testing conditions. In the SEM image of Figure 1, Al_2O_3 NPs are nearly spherical in shape. Al_2O_3 NPs appear to be agglomerated and have an approximate size of about 20 nm. Other characterization related to the shape of Al_2O_3 NPs was performed with XRD (Figure 2). In XRD analysis, the peaks were observed at 36.33, 45.07, 46.16, 62.03 and 66.70. *O. basilicum*, a well-known traditional herb in the Indian continent, has been included in several herbal preparations for the treatment of various diseases. Many types of *O. basilicum* and *O. sanctum* forms such as ethanoic extract, flavonoids, seed oil, phenolic compounds, root extract, leaf extract, aqueous extract, fixed oil, fresh leaf pulp and leaves were examined. Bone marrow radioprotection has promising results for chemo protection, hypoglycemic activity, and immune stimulatory effects (Prashar et al., 1994; Godhwani et al., 1998). The presence of xenobiotics and toxicants causes oxidative damage to cells and biomolecules. The negative effects of oxidative damage can be eliminated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) activities (Radotic et al., 2000; Lee et al., 2001; Liu et al., 2009). In this study, CAT activity remained stable in *O. basilicum* in response to Al_2O_3 NPs treatments. In addition, antioxidant activities of ethanol

extracts obtained from leaf and stem of *O. basilicum* plant were investigated by various methods. When investigations with *O. basilicum* were examined, it was seen that the determination of secondary metabolites, glycosylate and volatile aromatic compounds and their volatile components were limited to studies on determination of sulfur and nitrogen content and growing conditions. After treated *O. basilicum* with Al_2O_3 NPs, it was extracted with methanol in room conditions and its effects on H_2O_2 and CAT activities and MDA levels were examined (Table 3). Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation and shows an indication of free radical production and consequently tissue damage (Ohkawa et al., 1979). MDA is the result of peroxidation of fatty acids containing three or more double bonds. MDA level is used to evaluate lipid peroxidation. Increases in MDA levels were observed in Al_2O_3 NPs treated *O. basilicum*. The highest increase was observed concentration in 50 mg/L and this increase was statistically significant (P <0.05). In the application of 50 mg/L Al_2O_3 NPs, the MDA level increased by 7.409 times compared to the control. At 25 mg/L, the concentration of Al_2O_3 NPs increased 7.78 times compared to the control. At a concentration of only 75 mg/L, it was noted that the level of MDA was very low compared to the control. Furthermore, it was determined that CAT and H_2O_2 sweeping activity were the same for all Al_2O_3 NPs concentrations (P >0.05). According to these results, the highest tissue damage of Al_2O_3 NPs was 50 mg/L and 25 mg/L respectively. 100 mg/L caused very little tissue damage. At 75 mg/L, the MDA level was much less than the control group. In the application of 25 and 50 mg/L Al_2O_3 NPs which can be said to be actively penetrated in the cells. It can be said that high doses of 75 and 100 mg/L Al_2O_3 NPs do not cause tissue damage because they do not penetrate the cell due to aggregation. Because when nanoparticles form aggregates at high doses/concentrations, their passage to the cell is prevented. MDA and pigment analysis confirm each other. The chlorophyll content of leaf provides valuable information about the physiological state of plants. It makes it possible to evaluate chlorophyll content in leaves as fast and non-destructive *in situ*. Chlorophyll a and chlorophyll b are the pigments required to convert the light energy into stored chemical energy. The amount of sunlight absorbed by the leaf is a function of the photosynthetic pigment content. Thus, the chlorophyll content can directly determine the photosynthetic potential and primary production (Curran et al., 1990, Filella et al., 1995). In addition, chlorophyll indirectly estimates the state of the nutrient because a large part of the leaf nitrogen is incorporated into the chlorophyll (Filella et al., 1995, Moran et al., 2000). Moreover, the content of leaf chlorophyll is closely related to plant stress and aging (Merzlyak and Gitelson 1995; Merzlyak et al., 1999). The pigment content of the sweet basil leaves varied considerably. (Figure 3). In this study, chlorophyll a content was examined as an indicator of productivity in basil plant. Accordingly, chlorophyll a content is low in low Al_2O_3 NPs concentrations compared to the control group; high Al_2O_3 NPs concentrations were observed to be high. Compared to the control group, it decreased 61.7% in 25 mg/L and 13.5% in 50 mg/ L. Productivity is expected to decrease with increasing concentration of toxicants compared to conventional toxicants. However, in this study, productivity increased with increasing Al_2O_3 NPs concentration and low concentration productivity decreased significantly. This may be explained by the tendency of aggregation which is one of the unique physicochemical properties of the nanoparticles. Nanoparticles indicate high affinity to each other and form an aggregation. Thus, the nanoparticle cannot penetrate the cell by increasing its size. Also, as mentioned above, the MDA level used as an indicator of cell damage showed compatibility with the content of chlorophyll a. In other words, the level of chlorophyll at the concentration of Al_2O_3 NPs (25 and 50 mg/L) with the highest MDA levels was quite low. On the contrary, chlorophyll a level was high at concentrations (75 and 100 mg/L) with low levels of MDA.

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