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2,4-Dichlorophenoxyacetic Acid Loaded Polymeric Nanoparticle Synthesis and Its Effect on Biomass of *Medicago sativa* Cell Suspension Cultures

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ABSTRACT

Nanoparticular systems, which have made great advances with the development of nanotechnology, have been used extensively recently in pesticide, herbicide plant growth regulators, fertilizer applications, gene transfer technologies and agriculture. It was aimed to synthesize and characterize 2,4-D loaded PLGA nanoparticles and investigate their biological activity in comparison with its free form. Here, the effects of 2,4-D loaded poly (lactic-co-glycolic) acid (PLGA) nanoparticles on biomass in Medicago sativa cell suspension cultures were investigated. Single emulsion solvent evaporation method is used in nanoparticle synthesis. As a result of the characterization of nanoparticles, 63.82% encapsulation efficiency, 60.73% reaction efficiency and 10.51% drug loading capacities were calculated. Particle size was measured as 181.7 ± 3.74 nm, zeta potential -18.3 ± 1.48 and polydispersity index as 0.081. Compared with the free 2,4-D molecule, it was observed that the addition of 2,4-D to the medium using the nanoparticles drug release system increased the growth of plant cells and the yield of biomass in *M. sativa* cell suspension cultures.

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Introduction

Herbicides disrupt the hormonal balance that regulates plant metabolism, such as cell division, cell growth, protein synthesis, and respiration. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetically available, hormone-like, translocable, systemic selective herbicide [1]. It has an enzyme activity in plant metabolism that influences plant growth [2]. For this reason, it is used as a synthetic auxin and helps to induce rooting of scions and fruit drop [3]. Additionally, it ensures to regeneration of callus and root formation at low doses in *in vitro* cultures. Gopi and Vatsala stated that, maximum callus growth was

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obtained from 2,4-D, compared to 1-Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA) in *Gymnema sylvestre* [4]. In a study conducted on effects of kinetin and 2,4-D over callus formation in various mediums, kinetin was not effective alone while 2,4-D induced callus regeneration were reported [5]. Malik et al. stated that high 2,4-D levels inhibit callus proliferation, but low concentrations of 2,4-D involve morphogenesis [6]. Besides these positive effects of 2,4-D, it has toxic effects at higher concentrations was reported by another research for rooting of vine rootstock cuttings [7]. Additionally, 2,4-D concentration affect germination and initial development of *Regnellidium diphyllum* Lindman (Monilophyta, Marsileaceae). The development ratio of megaspores were high at lowest concentration of 2,4-D, it was negatively affected as the concentration increased [8]. Overall, previous studies show that the concentration of 2,4-D has important roles in plant growth and development.

Cell suspension cultures are one of the methods used for the production of medically and economically valuable secondary metabolites [9]. The production of secondary metabolite from whole plants is limited owing to cultivation and environmental limitations. The cells in the suspension medium have many advantages since they are physiologically more homogeneous and controllable. Plant cell cultures may also use as model systems for the study of metabolite pathways because the initiation of cell growth in suspension culture leads to rapid increase of biomass and a condensed biosynthetic cycle. Whereas, secondary metabolite synthesis may occur in specific cell and organ types and at a certain time period in whole plants. Because of these advantages, cell suspension cultures are frequently used in plant tissue culture studies [10].

In recent years, there has been a significant increase in studies on nanoparticular systems in areas such as medicine, food and agriculture. In these systems, the continuous release of active chemicals from nanomaterials can increase the effectiveness of them by offering better results with lower doses and number of applications [11]. Although different nanoparticular systems (metalic, polymeric, protein etc.) have various features, especially polymeric nanoparticles with high stability in biological fluids are preferred. This is because of polymeric nanoparticles which are obtained from biodegradable materials and it makes them possible to release the active ingredient in the target tissue by biodegradation. The Poly- (D,L-lactic-co-glycolic acid) (PLGA) is the most commonly used polymer in the production of polymeric nanoparticles. It has been determined that

the systems created by loading the active substances into the PLGA nanoparticle have increased the pharmacokinetic properties, therapeutic indices and biocompatibilities of the active substances compared to the free form [12-15].

European Medicine Agency (EMA) and Food and Drug Administration (FDA) approved that PLGA nanoparticles can be used for the preparation of DNA, RNA, peptide and protein carrier systems in many different structures. Additionally, having long-term clinical experience, being biocompatible and biodegradable are among the advantages of PLGA [13, 16]. For these reasons, PLGA is accepted and used as the gold standard of biodegradable polymers [17].

Although there are many studies about nanoparticular systems of PLGA [11, 18, 19], a controlled delivery system where 2,4-D is used as an active ingredient have not been found in the literature.

Thus, in this study whose originality was demonstrated, the characterization and *In vitro* release of the 2,4-D loaded nanoparticular system which prepared by a single emulsion solvent evaporation method using PLGA copolymer was investigated. Then, the optimized nanoparticles were added to the nutrient medium in *Medicago sativa L.* suspension cultures and their effect on time-dependent biomass yield was investigated.

Materials and Methods

Materials

PLGA (lactide:glycolide = 50:50; inherent viscosity 0.45–0.60 dL/g, Mw ~ 38-54 kDa,), polyvinyl alcohol, 2,4-dichlorophenoxyacetic acid, ethanol, dichloromethane (DCM), NaCl, NaOH, HCl, $Na_2HPO_4.2H_2O$, NaH_2PO_4, Murashige and Skoog (MS) medium were purchased from Sigma Aldrich (St. Louis, USA). All the chemicals and solvents used in nanoparticle preparation were of analytical grade and used without further purification.

Methods

Preparation of nanoparticles

2,4-D loaded PLGA nanoparticles were prepared with a single emulsion solvent evaporation method according to the literature [20]. Firstly, 2,4-D and PLGA were dissolved in ethanol and DCM, respectively. After that, solutions were mixed for the preparation of the homogeneous mixture. Mixture was added to 3% (w/v) polyvinyl alcohol (PVA) solution drop by drop. Sonication was done to emulsify the aqueous phase and the organic phase. The emulsified solution was added to 0.1% (w/v) PVA solution so

that nanoparticles were formed in the solution. Organic solvents (DCM and ethanol) were evaporated incubating at room temperature overnight, then the nano-suspensions were centrifuged at 10,000 g for 40 minutes at 4 °C (Beckman Coulter Allegra X-30R Centrifuge, Germany). The supernatant was collected and the nanoparticles were washed three times with 35 mL of distilled water. The collected supernatants were used in indirect measurement to determine encapsulation efficiency. The prepared 2,4-D loaded PLGA nanoparticles were freeze-dried for 48 hours at 0.01 mbar at -70 °C without any cryoprotectant. All lyophilized nanoparticles were stored at -80 °C until use.

UV-Vis spectroscopy, size and zeta potential, FT-IR and *In vitro* drug release analyses were used for characterization of nanoparticles. Reaction yield (RY), encapsulation efficiency (EE) and drug loading capacity (DL) were determined indirectly by spectrophotometric UV measurement of the supernatant obtained after centrifugation. Particle size and zeta potential values of 2,4-D loaded PLGA nanoparticles were determined by dynamic light scattering and electrophoretic light scattering techniques, using Zetasizer (Nano ZS, Malvern Instruments, UK). Functional groups present on the surface of nanoparticles were analyzed by Fourier-transform infrared spectroscopy in universal attenuation total reflectance (ATR) mode. *In vitro* release studies were performed at 37 °C at pH 7.4 during 30 days.

Characterization of 2,4-D loaded PLGA nanoparticles

Reaction yield (RY), encapsulation efficiency (EE), drug loading capacity (DL)

Reaction yield is obtained by calculating the ratio of solid nanoparticles obtained by freeze-drying to the total amount of 2,4-D and PLGA used. It is calculated as given in Equation 1. The amount of 2,4-D in the nanoparticle was found by subtracting the amount of 2,4-D found in the supernatant from first 2,4-D amount used. Encapsulation efficiency was calculated with indirect method according to Equation 2. Drug loading capacity was calculated with the ratio of the amount of substance in the nanoparticle calculated by the encapsulation efficiency to the total amount of nanoparticle obtained (Equation 3).

$$Reaction\ Yield(\%) = \frac{Obtained\ NPs\ (mg)}{Amounts\ of\ Total\ Material\ (2{,}4D+PLGA)}\ x\ 100 \qquad (1)$$

$$Encapsulation\ Efficiency\ (\%) = \frac{2{,}4-D\ into\ NP\ (mg)}{Amounts\ of\ Total\ 2{,}4-D\ (mg)}\ x\ 100 \qquad (2)$$

$$Drug\ Loading\ (\%) = \frac{2{,}4-D\ into\ NP\ (mg)}{Amounts\ of\ Total\ NP\ (mg)}\ x\ 100 \qquad (3)$$

Particle size and zeta potential

The dynamic light scattering and electrophoretic light scattering techniques were used for the analysis of particle size and zeta potential values of 2,4-D loaded PLGA nanoparticles, using Zetasizer (Nano ZS, Malvern Instruments, UK) [21]. 2,4-D loaded PLGA nanoparticles solution diluted with ultra-pure water was used for the measurements. Each measurement was done in triplicate at 25 °C.

FT-IR analysis

Fourier-Transform Infrared spectroscopy was used for the functional groups analysis on the surface of nanoparticles by in universal attenuation total reflectance (ATR) mode [22]. The FTIR spectra of 2,4-D, PLGA and 2,4-D loaded PLGA nanoparticles were obtained with 32 scans per sample and resolution of 4 cm^{-1} in the region of 650 to 4000 cm^{-1}.

In vitro drug release study

The *in vitro* release study of 2,4-D loaded PLGA nanoparticles was carried out according to the modified version of the dissolution method [23]. Firstly, 5 mg of lyophilized 2,4-D loaded PLGA nanoparticles were suspended in 10 mL of phosphate buffer saline at pH 7.4 by vortexing until particles were fully dispersed. 10 mL samples were collected at a specific period of time then, centrifuged at 10,000 g, +4 °C for 20 min. Supernatants were analyzed by UV-spectrophotometer for the measurement of 2,4-D per mL. The 10 mL fresh buffer was added to the solution after each sampling.

Callus culture of alfalfa

The surface sterilization of alfalfa seeds was performed by using 50% commercial bleach (5% (v/v) NaOCI) for 10 minutes and then rinsing with sterile distilled water three times. After that, they were placed on MS medium (Murashige & Skoog) with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.6 before sterilization at 121 °C for 25 min. Hypocotyls were removed from 7-day-old aseptic seedling and used as the explant. The explants (0.5-1.0 cm) were cultured on MS medium with 3 mg/L 2.4-D and 0.5 mg/L BAP for callusing. Cultures were incubated at 25 °C in the dark. After 6 weeks, cell suspension cultures were initiated with 1 g of friable callus.

Effect of 2,4-D loaded PLGA on cell biomass

Callus were transferred to 30 mL MS liquid medium containing 4 mg/L 2,4-D or 2,4-D loaded PLGA nanoparticles in Erlenmeyer flasks. The cultures were shaken continuously at 110 rpm in an orbital shaker and were kept in the growth chamber at 24±2 °C in the

dark. Twelve replicates were used for each treatment. The cell biomass was determined during 28 days by measuring every 7 days. Three replicates were used to each measurement. The cell biomass yield was measured in term of fresh weight and was determined using the formula in Equation 4.

$$The\ cell\ biomass\ yield = \frac{Final\ weight - Initial\ weight}{Initial\ weight}\ x\ 100 \quad (4)$$

Statistical Analyses

The data were presented as means ± standard deviation (SD) of at least three separate experiments performed in triplicate. Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA).

Results

Nanoparticle analysis

Characterization studies of synthesized and optimized nanoparticles were carried out by size analysis, zeta potential measurement, FT-IR and *in vitro* release studies. Particle size distribution and zeta potential measurement of the 2,4-D loaded PLGA nanoparticles were performed with the Zeta Sizer (Malvern ZEN 3600 Nano iS10). The average particle size of the nanoparticles were measured as 181.7±3.74 nm, the mean zeta potential value was -18.3±1.48 mV and the multiple distribution indices determining the size distribution were measured as 0.081. EE was found as a 63.82%, RY 60.73% and DL 10.51 %. Table 1 shows the results regarding the optimized 2,4-D loaded nanoparticles.

Table 1 Physicochemical characterization results of 2,4-D loaded PLGA nanoparticles

	RY (%)	EE (%)	DL (%)	Size (nm)	Zeta (mV)	PDI
2,4-D Loaded PLGA Nanoparticles	60.73	63.82	10.51	181.7±3.74	-18.3±1.48	0.081

FT-IR spectra of free 2,4-D molecule, PLGA and 2,4-D loaded PLGA nanoparticles were plotted to determine polymer interactions with 2,4-D in the nanoparticle formation process. As a result of the examination of the FT-IR spectra , FT-IR spectra of the nanoparticles were similar to the PLGA spectrum and there is no extra band (belonging

to any adsorption or a newly formed bond) observed. The result indicate that the 2,4-D molecule is effectively loaded to nanoparticles (Figure 1).

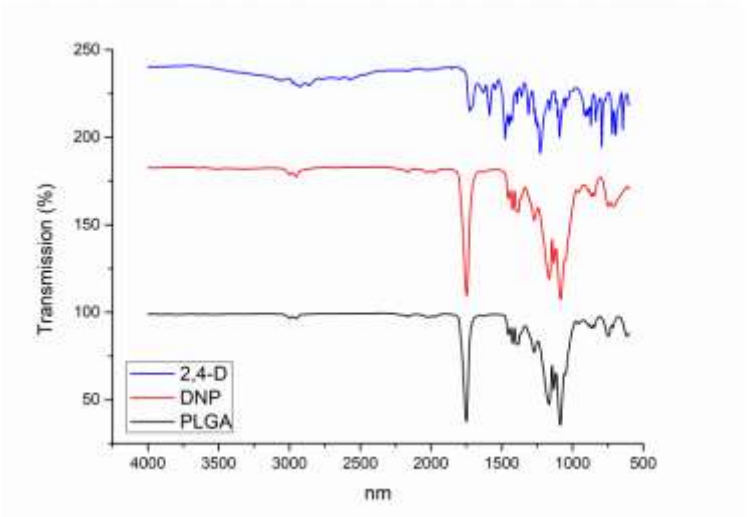

Figure 1 FT-IR Spectra of 2,4-D, PLGA and 2,4-D loaded PLGA nanoparticles (DNP)

The release experiment of the nanoparticles, which were suspended in PBS buffer (pH 7.4) and allowed to shake horizontally at 200 rpm at 37 °C, were performed for a period of 30 days. The samples were centrifuged at 10,000 g for 20 minutes at certain times and the 2,4-D amount released calculated by reading the UV values of the upper phases at 284 nm in the UV spectrophotometer. As a result of the release experiments it is observed that the 2,4-D contained in the nanoparticles was released slowly and in a controlled manner and released a total of 37.10% after 30 days.

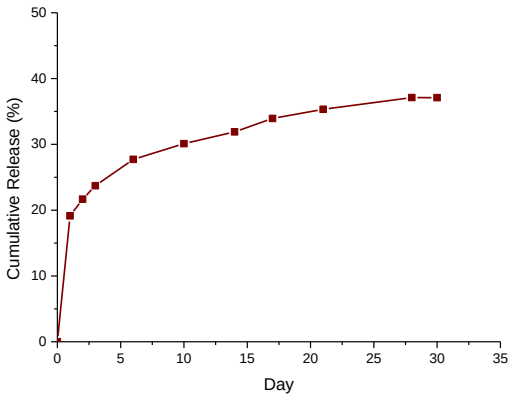

Figure 2 *In vitro* drug release of 2,4-D loaded PLGA nanoparticles

Preparation of nanoparticular systems was carried out by single emulsion (o/w) solvent evaporation method [24]. Reaction yield (RY), entrapment efficiency (EE), drug loading

(DL), particle size (Z-Ave), polydispersity index (PDI) and zeta potential (mV) analyzes were examined, and FT-IR and *in vitro* release analyzes were performed for characterization. Various syntheses have been made for nanoparticle characterization and used in tissue culture studies by mixing particles of similar properties that provide ideal results for all parameters. In optimized nanoparticles; RY 63.16%, EE 46.26%, DL 10.51%, size 181.7 nm, zeta potential value -18.3 mV and PDI value as 0.081 was determined. The analysis of the FT-IR spectra obtained, it was observed that the optimized nanoparticles have PLGA properties and this result shows that, the 2,4-D is effectively encapsulated in to the nanoparticular system. In the emission study, it was observed that the 2,4-D emission occurred slowly for 30 days and at the end of this period, it released 37.10%.

Evaluation of cell biomass yield

One of the most important disadvantages of PGR application in agriculture is that these compounds degrade rapidly under high light and temperature conditions, leading to a loss of biological activity. Additionally, most of the PGRs can be phytotoxic when used in high concentrations. Therefore, development of controlled release systems for PGRs are extremely important [25]. In this study, the effects of 2,4-D loaded PLGA nanoparticles on biomass yield in alfalfa suspension cultures were evaluated. Changes in biomass yield in cell suspension cultures were determined 4 times (every 7 days) until day 28. Biomass yields were calculated using the formula in Equation 4 by data fresh weight of biomass obtained from cell suspension culture every 7 days. As a result of the first week of the treatment, the average biomass yield in MS medium containing free 2,4-D was measured as 104%. The average biomass yield was measured as 148% in MS medium with 2,4-D loaded PLGA nanoparticles. At the second week of culture, the average amount of biomass in MS medium with free 2,4-D was 154% while biomass yield in 2,4-D loaded PLGA nanoparticles was 231%. Biomass yield values obtained at the end of the third week was 113% for free 2,4-D; It has been determined as 264% for 2,4-D loaded PLGA nanoparticles treatments. The biomass yields in the fourth week were determined as 138% and 304% in free 2,4-D and 2,4-D loaded PLGA nanoparticles treatments, respectively.

The results showed that the increase of biomass yield in free 2,4-D reached the highest value in the 2nd week. The increase of biomass was lower in the 3rd and 4th weeks compare with the 2nd week. However, the increase of biomass yield has continued in 2,4-

D loaded PLGA nanoparticles applications regularly. As a result of these calculated values, it was determined that the growth rate of 2,4-D loaded PLGA nanoparticles was more effective than free 2,4-D (Fig 3).

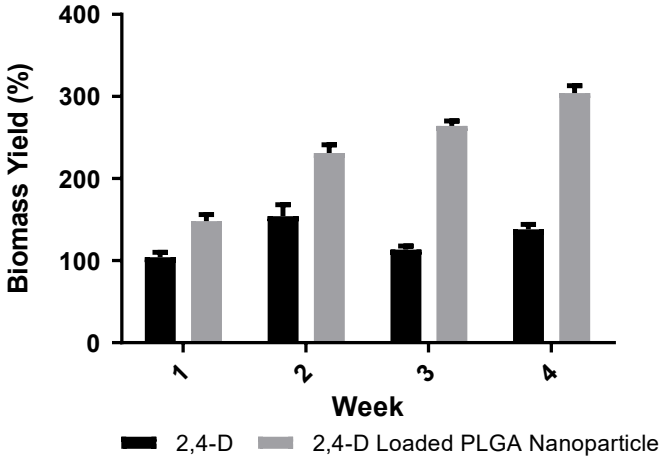

Figure 3 Effects of 2,4-D and 2,4-D loaded PLGA nanoparticles on *M. sativa* biomass

During cell culture measurements, it was observed that the bulk of the cells obtained from the medium with free had a darker colour (Fig 4). Supernatant could be darker due to the cell death. While the cells were slowly exposed to 2,4-D in the nanoparticle release system, they were exposed directly 2,4-D in the medium with free 2,4-D. It is known that high concentrations of 2,4-D may have a toxic effect. The results thought that free 2,4-D caused cell death in MS medium.

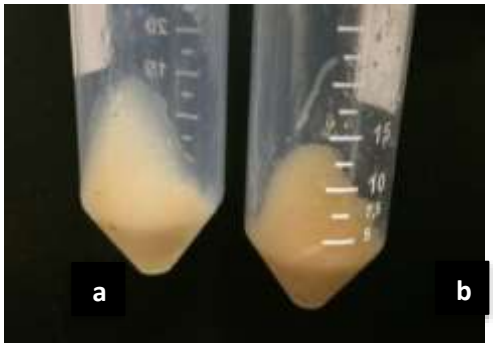

Figure 4 Biomasses obtained from media containing 2,4-D loaded PLGANP (a) and free 2,4-D (b)

Discussion

As it is known, polymeric nanoparticles have advantages such as increasing the solubility of the hydrophobic molecules in water, increasing their biocompatibility, enabling them to be active in the biological system for a longer time and at lower dose [26]. Due to these

advantages, it has been reported that many hydrophobic molecules are loaded into nanoparticular systems, thus increasing their biological activities [15, 27-29]. There are many studies on nanoparticular systems in mammalian cell cultures and nanoparticles have been used quite successfully until now in mammalian cell cultures and drug targeting systems [30, 31]. Recently, applications of nanoparticles on plants have gained popularity. The use of nanotechnology in agriculture aims to minimize the use of herbicides, pesticides and fertilizers while increasing agricultural production [25]. By encapsulating various chemicals used in agriculture into polymeric nanoparticles, undesirable toxic effects on organisms can be reduced and the thermal and photochemical stability of the encapsulated active ingredient can be increased [32]. Polymeric nanocarriers are able to control the delivery of chemicals to plants and reduce excess run-off [33]. In addition, nanoparticles are ideal materials due to their low cost, biocompatibility and biodegradability [25].

Callus culture is considered as an important starting material for the production of large amounts of biomass in plant biotechnology studies [34]. However, in some studies on PGRs, it is known that PGRs given after optimum dose negatively affect biomass. For example, Ma et al. reported the negative effects of GA3 over 50 μM in *Leymus chinensis* [35]. Therefore, the use of various PGRs in nanocarriers can positively affect plant growth and biomass by preventing the achievement of supra-optimal levels by enabling the slow and sustained release of the active substance [11].

The effect of various plant regulators and particulate systems on cell culture studies in recent years has been studied. In a study using methyl jasmonate-loaded PLGA nanocarriers to improve natural defences in *Vitis vinifera*, Chronopoulou et al. showed that methyl jasmonate-loaded PLGA NPs react faster compared to free MeJA, encapsulation in PLGA NPs significantly increased MeJA cell uptake and activation of MeJA-induced responses [36]. In another study where PLGA-block-PEG Copolymers were used as carriers for controlled release of herbicides into the soil, in the 3-month release test, it was observed that almost 90% of the loaded herbicides were slowly released into the soil. This is very important for agriculture because the average growing season of cultivated plants is about a few months [37]. Studies on *Phaseolus vulgaris* have been performed in chitosan-based nanoparticles prepared as carrier systems of GA3. In the study, GA3, Alginate/Chitosan-GA3 NP and Chitosan/Tripolyphospathe-GA3 NP

were compared and ALG/CS-GA3 showed stronger effects in terms of leaf development and carotenoid levels [38]. Pereira et al. synthesized GA3 nanoparticles with γ-PGA and Chitosan as a plant hormone carrier. The nanoparticular system was more effective than free hormone in *Phaseolus vulgaris* seeds and accelerates germination within 24 hours after treatment and increases leaf area and root development (including the formation of lateral roots) [39].

There are a lot of studies on the loading of molecules with low solubility and stability in the physiological environment in to the nanoparticular systems [26, 40-43]. These systems can improve the distribution of the molecule more effectively and more efficiently in nanoparticles that can be controlled and can release for a long time. A few promising studies have evaluated the use of PLGA nanoparticle in plants. Atrazine (ATZ), was encapsulated into PLGA to prevent damage to the environment. When the release profiles of ATZ loaded PLGA-NPs were examined, it was observed that they showed a much slower release compared to the pure herbicide [19]. In another study with ATZ, potato plants were used as biological models and herbicidal activity of designed PLGA nanoherbicides were evaluated. As a result of in vitro findings, it was determined that as the ATZ concentration increased in PLGA nanoparticles, potato plants experienced a significant decrease in root length, fresh weight, dry weight and leaf number. It was observed that the root length was affected the most among these parameters. Thus, it has been proposed that PLGA nanoherbicides can be used as an alternative method to prevent weed growth [44]. Tong et al. stated that nanoparticles obtained in the formulation in which metolachlor was encapsulated into mPEG-PLGA increased the solubility of hydrophobic metolachlor in water and did not contain any active substance on its surface. Nanoparticular systems have been proven to have a higher biological effect on *Oryza sativa* and *Digitaria sanguinalis* compared to free metholachlor. In addition, in the cytotoxicity test, it was observed that the NP toxicity decreased on the preosteoblast cell line [18]. These studies showed that polymeric nanoparticles caused less environmental damage when used as a pesticide carrier and contributed to plant growth by eliminating various damages of herbicides.

Considering unique properties of nanoparticles and the lack of nanoparticular systems with 2,4-D in the literature; 2,4-D, which is a water-insoluble and toxic molecule, was added to nanoparticular systems, thus making it soluble in tissue culture studies. At

present study, free 2,4-D and 2,4-D loaded PLGA nanoparticles were applied to *M. sativa* cell cultures and their effects on biomass yield were compared. The addition of 2,4 D loaded PLGA nanoparticles to the medium was much more effective compared to the free 2,4-D added medium, thanks to the controlled release of 2,4-D in the particulate system.

Conclusion

In this study, it is aimed to reduce the adverse effects of 2,4-D molecule, which is known to show a high concentration level of toxic effects, by nanoparticular systems. We also intended to increase its effectiveness by remaining intact for a longer time thanks to the controlled release mechanism of the substance. It is known that nanoparticle release systems increase the effect of active subtances on cells. However, a study on the application of 2,4-D using nanoparticular release systems in plant cell cultures has not been reached. In this study, the effect of applying 2,4-D with nanoparticle release systems to plant cell cultures was demonstrated for the first time. After reaching the optimized formulation of the 2,4-D loaded PLGA nanoparticles system, the effect of 2,4-D loaded PLGA NPs on plant cell growth was investigated. At the end of the study, it was observed that the addition of 2,4-D to the medium using the nanoparticles drug release system was increased the amount of cell biomass. It has been observed that the particulate system was much more effective than the free substance at the study.

It is thought that the optimized system, which was loaded into the particular system and whose water solubility increased and positive results were obtained in plant cell culture studies, may be a reference for drug delivery systems. These systems can be used as an alternative method for the addition of compounds with a high concentration of toxic effects to the medium. In nanoparticle systems, the amount of oscillation can be adjusted and time-dependent manipulation of cells can be performed without the need for subculture.

Abbreviations

2,4-D; 2,4-Dichlorophenoxyacetic Acid, PLGA; Poly (lactic-co-glycolic) acid, EE; Encapsulation efficiency, RY; Reaction yield, DL; Drug loading, FT-IR; Fourier-transform infrared spectroscopy, MS medium; Murashige and Skoog medium, DNP; 2,4-D loaded PLGA nanoparticles.

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