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Polymerization degree-dependent changes in the effects of *in vitro* chitosan treatments on photosynthetic pigment, protein, and dry matter contents of *Ipomoea purpurea*

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Abstract

Morning Glory (*Ipomoea purpurea* (L.) Roth.) is a climbing plant known for its ornamental properties and ease of cultivation in temperate climates. Quality and colour of flowers and leaves, especially in the production of ornamentals, are important parameters both for producers and for customers. This study aimed to investigate the changes in photosynthetic pigment, protein and dry matter content of *in vitro*-propagated *I. purpurea* following chitosan treatment with different polymerization degrees (DP) and to determine the indirect effect of this biopolymer on leaves of the plant. Nodal explants of *I. purpurea* were cultured in medium supplemented with 5, 10 and 20 mg L^{-1} concentrations of a chitosan oligomers mixture with a variable degree of polymerization (DP) ranging from 2 to 15 or chitosan polymer with DP of 70. It was found that both oligomeric and polymeric chitosan treatments increased chlorophyll-a contents in the leaves when compared to the chitosan-naïve control group. Polymeric chitosan stimulated chlorophyll-b and carotenoid synthesis more effectively than the oligomer mixture. Also, 10 mg L^{-1} polymeric chitosan better triggered total protein production and plant dry matter content in *I. purpurea*. The results of this study showed that, due to their stimulatory effects on photosynthetic pigment, protein and plant dry matter production, chitosan oligomers at low concentration and polymers at moderate concentration might be considered as safe and natural biostimulants for ornamental plants which could affect the plant's attractiveness and commercial success.

Keywords: Chitosan, dry matter, photosynthetic pigments, polymerization degree, protein

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Introduction

Ornamental plants are usually preferred because of their colorful and attractive flowers, leaves and general appearances. Thus, visual appearance is the key factor in the commercialization of ornamentals, since these factors greatly affect consumers' purchase likelihood (1). Generally, synthetic plant growth regulators (PGRs) are used by many growers for various purposes, especially to enhance plant characteristics such as sprouting rate, plant height, lateral branching and rooting (2,3). However, the increasing preference among consumers for eco-friendly and biodegradable alternatives to PGRs impels growers to find sustainable natural alternatives to PGRs (4).

Chitosan, which has been shown to be a natural alternative to commonly used synthetic PGRs (5), is the deacetylated form of the biopolymer of chitin. Chitin is found in the shells of members of the *Crustaceae* and the carapaces of insects, in the cell walls of fungi and in some algae (6). Chitosans have been used to enhance seed germination rate, to induce lateral branching and earlier flowering and to extend vase-life in ornamental plants. In these reports, *in vitro* effects of chitosans have been mainly investigated based on morphometric parameters such as plant shoot and root length, and the number of shoots, roots and leaves (5,7,8). Besides these parameters, basic physiological parameters such as photosynthetic pigments, protein, and relative water contents could also be useful in giving an insight into the effects of different chitosans, since these parameters are related to the visual appearance of ornamentals.

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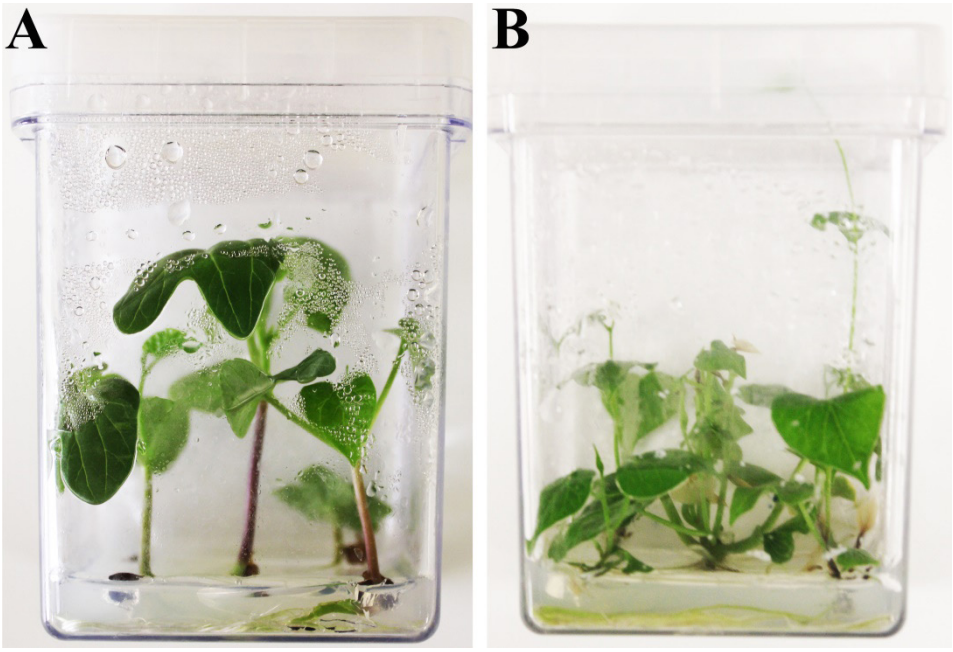


Figure 1. *In vitro* culture establishment of *Ipomoea purpurea* (A) Donor plantlets obtained through seed germination. (B) General view of the cultures during the shoot multiplication phase.

Polymerization degree (DP), which represents the number of monomeric units in a polymer, and degree of acetylation (DA), that is the molar fraction of *N*-acetylated glucosamine, are two of the main molecular characteristics that affect the responses of plants to chitosans (9). Thus, the physiological effects of partial *N*-acetylated chitosan oligomers and polymers in plants should be investigated to elucidate these structure-function relationships. In order to facilitate this investigation, a widespread and easy-to-grow species should be employed in a study showing the polymerization degree-dependent changes in the effects of chitosan treatments on photosynthetic pigments, protein, and relative water contents.

Morning Glory (*Ipomoea purpurea*) is a climbing plant which is classified in the Convolvulaceae (Bindweed) family. *I. purpurea* has been cultivated in many countries for its ornamental merit. Morning Glory is popular among growers because of its strong spreading ability and ease of culture (10). Due to these properties, *I. purpurea* was used as a model ornamental plant in this study to show the *in vitro* effects of different chitosan treatments at different polymerization degrees on a range of physiological parameters.

Material and Methods

Plant material and culture establishment

The seeds of *Ipomoea purpurea* (L.) Roth supplied from Vilmorin Garden, Turkey, were cultured *in vitro* to supply an adequate number of plant material for shoot multiplication. Surface sterilization of the seeds was done in 1% (w/v) sodium hypochlorite (NaOCl) solution with gentle shaking for three minutes, as described by Acemi *et al.* (5). The seeds were then transferred onto Murashige & Skoog (MS) medium (11) and incubated for 30 days (**Fig. 1A**). At the end of the incubation period, nodal explants excised from regenerated shoots were multiplied in the same MS medium, supplemented with 0.5 mg L^{-1} kinetin (**Fig. 1B**). Nodal explants with at least one node were taken from the multiplied shoots and further cultured in MS medium, supplemented either with chitosan oligomer mixture, with DP ranging from 2 to 15 or polymer (DP 70) at three different concentrations (5, 10, and 20 mg L^{-1}) as previously described by Acemi *et al.* (5). The DA of each chitosan variant was 10%. The cultures were maintained at 23±1°C temperature under the illumination of 60 µmol m^{-2} s^{-1} photosynthetic photon flux density with a 16-hour photoperiod during the incubation period.

Chitosan characterization

The chitosan samples were prepared chemically from shrimp shell wastes. The polymeric chitosan was characterized using HP-SEC-RID-MALLS (12) while the oligomeric chitosan mixture was analyzed using MALDI-TOF-MS (13). ^{1}H-NMR was used to analyze DA (%) of the samples (14). The chitosan polymer and oligomers were characterized and provided by the Institute of Plant Biology and Biotechnology, University of Münster, Münster, Germany.

Leaf photosynthetic pigment quantification

Tissue samples were extracted using absolute acetone, and the extract was centrifuged at 6000 rpm for 10 min at 4 °C. The supernatants were then analysed spectrophotometrically. Chlorophyll a (C_a), b (C_b) and total carotenoid (C_{x+c}) quantities were calculated using the equation (1) described by Lichtenthaler (15). The data were expressed as mg g^{-1} dry weight (DW).

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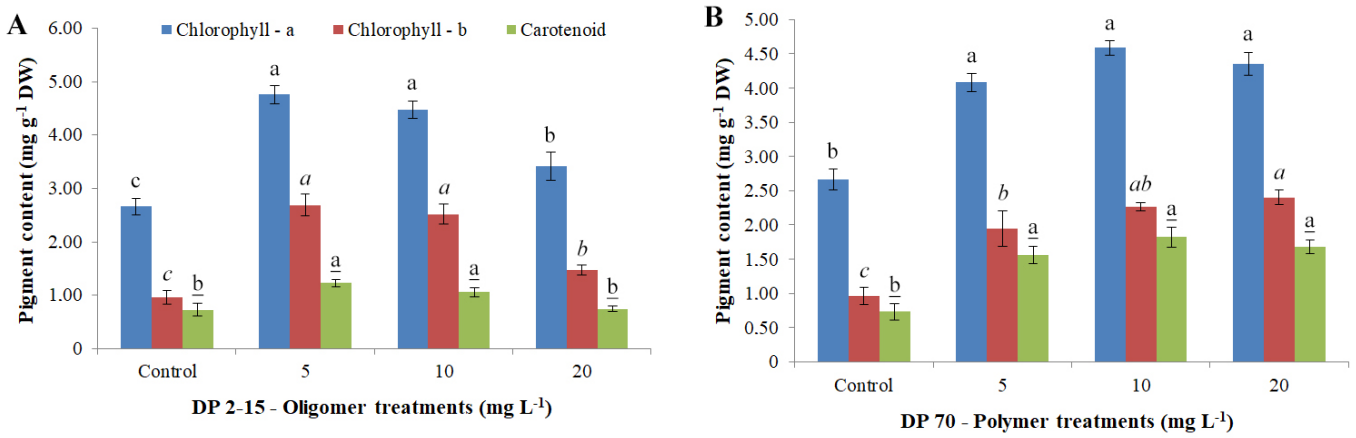


Figure 2. Concentration-dependent effects of chitosan oligomer mixture (A) and polymer (B) on photosynthetic pigment content in *in vitro*-propagated *Ipomoea purpurea* leaves.

(1) $$C_a = 11.24A_{661.6} - 2.04A_{644.8}$$
$$C_b = 20.13A_{644.8} - 4.19A_{661.6}$$
$$C_{x+c} = (1000A_{420} - 1.90C_a - 63.14C_b) / 214$$

Leaf total protein quantification

The extracts were prepared following the method of Bonjoch and Tamayo (16). Fully expanded and healthy leaves were sampled and transferred into a pre-cooled mortar. The samples (200 mg) were ground in a cold Tris buffer (0.05 M, pH 8.0) supplemented with 0.05 g polyvinylpyrrolidone (PvP). The final mixture was centrifuged at 15,000 g for 20 min at 4 °C. The quantity of total soluble proteins was estimated from supernatants according to the method of Bradford (17). The data were expressed as mg g^{-1} dry weight (DW).

Determination of total plant dry matter

The leaves, shoots, and roots were sampled (200 mg each) from individual plants having similar growth characteristics. The samples were then dried at 80°C in a hot-air oven for two days. The samples were kept in a desiccator before the measurement of dry weight. The data were expressed as dry matter percentage of weight.

Sample collection and statistical analysis

Only healthy leaves which showed visual uniformity in colour were employed, both in photosynthetic pigment and leaf total protein quantification experiments. Means were compared using Duncan's multiple range test at a significance level of $p<0.05$. Data were given as mean ± standard deviation (SD). The Statistical Package for Social Sciences (SPSS) version 22 software (IBM Inc., Chicago, IL., USA) was used for statistical analysis.

Results

Photosynthetic pigment contents after oligomeric chitosan treatments

The changes in the photosynthetic pigment contents after oligomeric chitosan treatments are shown in **Fig. 2A**. The highest pigment contents were observed in plants treated with 5 mg L^{-1} oligomeric chitosan. In general, all oligomeric chitosan treatments induced photosynthetic pigment production in *I. purpurea* compared to controls. Only carotenoid content from plants cultured with 20 mg L^{-1} oligomeric chitosan was found to be statistically the same as the control group. Chlorophyll a/b ratios for control plants and for increasing oligomeric chitosan treatments at 5, 10 and 20 mg L^{-1} concentrations were found to be 2.77, 1.77, 1.78 and 2.32, respectively. For oligomeric chitosan treatments >5 mg L^{-1}, the increasing concentrations caused a gradual reduction in the quantities of all the pigments (see **Fig. 2A**). However, the effect of the 5 and 10 mg L^{-1} treatments remained statistically the same.

Photosynthetic pigment contents after polymeric chitosan treatments

The changes in the photosynthetic pigment contents after polymeric chitosan treatments are shown in **Fig. 2B**. The highest pigment contents were observed in the plants treated with 20 mg L^{-1} polymeric chitosan. In a similar fashion to the oligomeric chitosan treatments, all polymeric chitosan treatments enhanced photosynthetic pigment production in *I. purpurea* compared to controls. There was a dose-dependent increase in photosynthetic pigment content in response to increasing polymeric chitosan concentrations. However, this increase was not statistically different between the concentrations for chlorophyll. Only chlorophyll b content in the plants cultured with 20 mg L^{-1} treatment was found to be statistically higher than that of the lower dose polymeric chitosan treatments. Chlorophyll a/b ratios for control and for increasing polymeric chitosan treatments (5, 10 and 20 mg L^{-1} concentrations) were 2.77, 2.09, 2.02 and 1.81, respectively.

Total soluble protein content

The changes in total soluble protein contents after chitosan treatments are shown in **Fig. 3**. The highest protein content was found in the plants treated with 10 mg L^{-1} polymeric chitosan. A gradual increase in total soluble protein content was ob-

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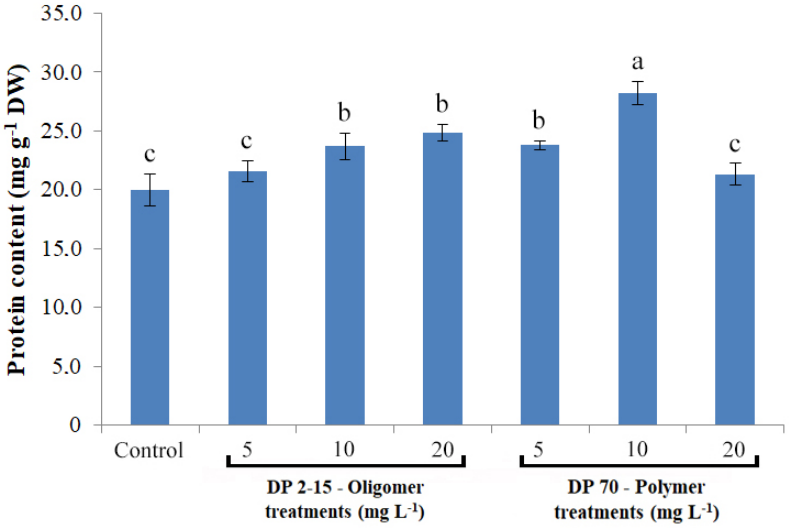


Figure 3. Effects of polymerization degree and treatment concentration of chitosans on soluble protein content in *in vitro*-propagated *Ipomoea purpurea* leaves.

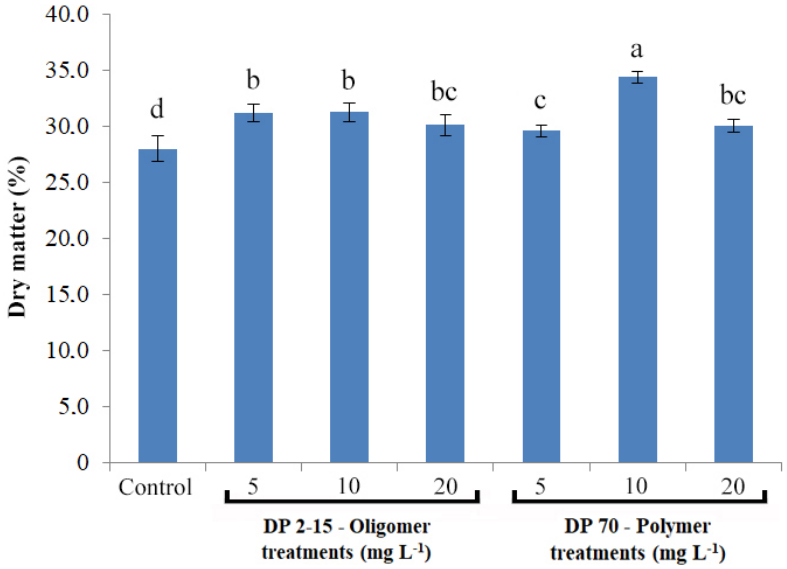


Figure 4. Effects of polymerization degree and treatment concentration of chitosans on dry matter production in *in vitro*-propagated *Ipomoea purpurea* plants.

served in response to increasing concentrations of oligomeric chitosan in the media. However, results of control and 5 mg L^{-1} oligomeric chitosan treatments were statistically same. Also, 10 and 20 mg L^{-1} oligomeric and 5 mg L^{-1} polymeric chitosan treatments yielded statistically the same total soluble protein content. The lowest soluble protein content was found in the control group of plants. Also, an evident decline in the protein content was observed in the plants grown in media supplemented with 20 mg L^{-1} polymeric chitosan. However, this was statistically the same as the controls.

Plant dry matter

The changes in the plant dry matter to fresh weight ratio after chitosan treatments are shown in **Fig. 4**. Dry matter production was induced after all chitosan treatments. However, the results for the plants grown in the medium with the 10 mg L^{-1} polymeric chitosan were found to be the highest. The results after oligomeric chitosan treatments at 5 and 10 mg L^{-1} were found to be statistically the same while a similar statistical result was found for the results of 20 mg L^{-1} oligomeric and 5 mg L^{-1} polymeric chitosan treatments.

Discussion

Chitosan is considered to be one of the inexpensive, easy-to-extract and safe biopolymers. Its chemical structure also makes it advantageous over other biopolymers because it enables designing the polymer for a wide range of applications from medical to horticultural purposes (18,19). The biologic effects

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of biopolymers may differ according to their chemical properties. Thus their DP and DA may have critical roles, especially in plant production (5). The name "chitosan" is a general name used to describe the deacetylated form of chitin. Therefore, chitosan includes various oligomers and/or polymers at different DP and/or DA (20). To elucidate the effects of different chitosan oligomers and/or polymers, the oligomers and polymers should be separated, purified and characterized prior to their application to plants. This study clearly shows the importance of varying chemical properties of chitosan oligomers and polymers and their effects on basic biochemical parameters in plants.

In our study, the production of photosynthetic pigments in *I. purpurea* was significantly different based on the application of different oligomeric and polymeric chitosans. Oligomeric chitosan mixture with DP between 2 and 15 increased photosynthetic pigment production more than polymeric chitosan with DP 70. Additionally, the results in the **Fig. 2A** indicate that oligomers at lower concentrations than 5 mg L^{-1} also might be efficient in terms of stimulating photosynthetic pigments. The photosynthetic pigment-inducing effect of chitosan has also been shown in *Brassica rapa* under Cadmium stress (21). Phothi and Theerakarunwong (22) reported that chitosan application improved photosynthesis rate in *Oryza sativa* plants. It should be noted that increased photosynthetic pigment concentrations are correlated with elevated photosynthetic performance in plants. Therefore, it can be said that both chitosan types can increase photosynthetic performance in *I. purpurea,* based on the findings of our study. This increase might be attributed to enhanced stomatal conductance, transpiration rate and/or cell size and number (23). In our experiments, the reduced amounts of photosynthetic pigments due to oligomeric chitosan application in increasing concentrations might be attributed to the effects of oxidative damage on the photosynthetic apparatus. This may be because chitosan has been reported to elicit plant defense responses (24), and it may trigger NADPH oxidase activity, thereby stimulating the production of H_2O_2. Thus chitosans may activate reactive oxygen species (ROS) signaling systems in plants (25). For example, chitosan application has been shown to induce endogenous H_2O_2 in *Capsicum annuum* (26). Considering the differences between photosynthetic pigment quantities in *I. purpurea* after oligomeric and polymeric chitosan treatments, we suggest that oligomers might tend to be more active in stimulating ROS production in *I. purpurea* compared to polymeric chitosans.

In plants, proteins play multiple roles such as regulators for plant development, catalysers in chemical reactions and promoters in cell membrane transport (27). Proteins also play a role in building intracellular structure and energy generating reactions, such as mitochondrial electron transport (28). In a recent report, chitosan application at 1% (w/v) concentration was shown to trigger reprogramming of protein metabolism with an increase of storage proteins in *Fragaria* × *annanasa* (29). In another report, Anusuya and Sathiyabama (30) found that chitosan treatment at 0.1% (w/v) increased protein content in *Curcuma longa* cv. Erode plants. The same researchers also reported that protein content correlated with chitinase enzyme activity, suggesting that chitosan can induce chitinase, chitosanase and β-1,3 glucanase activities as defense reactions in plants. Thus, the increase of leaf protein content in *I. purpurea* after chitosan treatment might be attributed to possible increases in these enzyme activities. However, it can be clearly seen that plant dry matter percentage also increased after chitosan treatments. Therefore, this increase could be due to increased synthesis of plant metabolites, such as lignin used in plant development. In this context, the stimulatory role of chitosan in lignin biosynthesis and cell wall lignification in plants has been demonstrated by Vasconsuelo *et al.* (31). Furthermore, chitosan may affect biosynthesis and translocation of carbohydrates during stimulated cell division and the synthesis of DNA and RNA (22).

In our previous study, the same chitosan oligomer mixture and polymer used in this study enhanced leaf number per shoot in *I. purpurea* (5). The finding of elevated photosynthetic pigment production after chitosan treatments in the present study may thus be due to improved plant development. Evidence supporting this supposition comes from earlier studies that showed that chitosan enhanced biomass in *Triticum aestivum* (32) and *Fragaria* × *annanasa* (33). Further evidence for this was reported by, El-Miniawy *et al.* (34) and Corsi *et al.* (35) who reported that chitosan application increased dry and fresh weights of roots in strawberry plants and kiwifruit, respectively. However, although some of them are statistically different than the control, the chitosan treatments induced close changes especially in protein and dry matter contents among the measured parameters.

Conclusions

This study shows that chitosan oligomers can be more efficient in the stimulation of photosynthetic pigment production when used at low concentrations in the culture medium. However, chitosan polymer at moderate concentration in the culture medium may better trigger total protein production and plant dry matter content in *I. purpurea*. In the ornamental plant industry, quality of leaves is strictly correlated with their colour richness and condition. The outcomes of the study suggest that, due to their stimulatory effects on photosynthetic pigment, protein and plant dry matter production, chitosan oligomers at low concentration and polymers at moderate concentration of not more than 10 mg L^{-1} in the medium may be considered as safe and natural biostimulants for ornamental plants which could increase the attractiveness of commercially valuable plants. However, this study should be conducted on other ornamental species to validate these conclusions.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Compliance

This article does not contain any studies involving human participants or animals performed by any of the authors.

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