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EFFECT OF NANO ZINC OXIDE ON THE CHLOROPHYLL CONTENT OF BEAN PLANTS UNDER DROUGHT STRESS

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Abstract. The mechanism by which nanoparticles are involved in drought tolerance responses in plants is complex and requires further investigation. Considering the important role of ZnO nanoparticles in plant defense systems, it is relevant to investigate the effects of these nanoparticles depending on the type of plants and their developmental stages. The results of our experiments have shown that ZnO nanoparticles can play a role in the drought resistance of bean plants. It is clear from the results that ZnO nanoparticles, when applied under normal irrigation conditions, have a positive effect on the height development of plants, reduce the vegetation period and keep the pigment content stable. The difference in the pigment content observed in our experiments during the growing season may be related to the formation of bean leaves in different layers at different times. It is interesting that in the plants subjected to drought stress (variant IV) where ZnO nanoparticles were applied, the pigment content remains stable, although the growth of the plants is not fast. , the growing season is shortened and pod formation occurs more quickly. In addition, Chl pigment degradation does not occur for a long time, the leaves retain their greenness.

Keywords: *Nanoparticles, beans, zinc oxide, drought, pigments, chlorophyll, carotenoids.*

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1. Introduction

More than 40% of the world's population is exposed to drought stress each year and by 2030, 7,000 million people will have to migrate from drylands. Among the environmental factors, drought affects the flora the most. Drought stress causes several physiological and biochemical effects as a factor that seriously affects the development of chicks. The most common responses of plants to drought stress are morphophysiological changes aimed at preventing water loss and reducing oxidative damage (Serraj *et al*., 2002). With the closing of the stomata in the leaves, the transpiration process is weakened, the transfer of CO_2 gas to the Calvin cycle is sharply reduced and as a result, the formation of biomass is weakened. Studies show that severe drought reduces the dry weight of roots and shoots, the synthesis of chlorophyll pigment in leaves and the relative water content of plants (Alaei *et al*., 2013). Reactive oxygen species (ROS) increase in cells, resulting in peroxidation of lipids in membranes and increased leakage of electrolytes. To overcome these damages, the amount of proline increases for sub-cellular stabilization and osmotic regulation in the cell cytosol (Ashraf

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et al., 2007). The increase in the concentration of essential oils is compensated by the decrease in biomass and as a result, their amount is the same in drought-exposed and normally watered plants (Selmar *et al*., 2013).

Therefore, plants use several different mechanisms to cope with drought, synthesizing more active forms of oxygen, synthesizing stress hormones such as ethylene and abscisic acid, changing root and shoot morphology, closing stomata and reducing chlorophyll synthesis (Etesami *et al*., 2015; Chiappero *et al*., 2019; Bhat *et al*., 2021). These mechanisms create short-term and long-term reactions in plants. When the duration of stress is short, the reactions are also short-term. During some short-term reactions, carbon assimilation is reduced, stomata are closed, osmotic regulation and growth is weakened, hydraulic changes occur and responses are generated such as the generation and transmission of signals indicating cell desiccation. These reactions do not cause serious consequences in plants if normal conditions are quickly restored. However, during long-term stress, irreversible changes occur in plants and eventually, they die (Kaur & Asthir, 2017)

Recently, the application of nanotechnology in plant physiology has made it possible to gain important achievements in agricultural practice. The application of nanotechnology in the fight against abiotic stresses that cause serious problems in the productivity of plants, including drought, has led to new approaches. The number of studies on the fact that nanoparticles from the specific materials of nanotechnology play an important role in increasing the drought resistance of plants due to their different physicochemical properties (Saxena *et al*., 2016) is increasing day by day. The use of nanoparticles to increase the resistance of plants to drought is a new approach and has been applied successfully. Silicon, silver, iron, titanium dioxide, copper, manganese, aluminum, etc. nanoparticles have a positive effect on several important processes in plants, increase the intensity of photosynthesis, reduce the amount of malondialdehyde, increase the relative amount of water, improve the root and sprout systems and accelerate the development of plants (Ashkavand *et al*., 2018). The mechanism by which nanoparticles are involved in drought tolerance responses in plants is complex and requires further investigation. However, several studies have shown that nanoparticles affect aquaporins, which regulate the water regime in plants and seed germination. As a result, it increases the water and nutrient demand of seeds during drought stress (Li *et al*., 2020). The role of nanoparticles such as TiO_2, ZnO, Fe_2O_3, Fe_3O_4, CuO, MnO in drought resistance has been widely studied. TiO_2 nanoparticles increase the rate of photosynthesis due to their characteristic photocatalytic properties. Ze et al. (2013) showed in their experiments that TiO_2 nanoparticles increase the ability of chloroplasts to absorb light, regulate the distribution of light between photosystems I and II, accelerate the conversion of light energy into electrical energy and increase the intensity of photosynthesis by affecting water splitting and oxygen release.

Some experiments have shown that nanoparticles increase the concentration of ROS, resulting in cytotoxic effects during abiotic stresses in plants (Khan *et al*., 2017). However, in low doses, nanoparticles play an important role in the fight against drought stress and other abiotic stresses in plants. Nanoparticles strengthen antioxidant systems, cause the formation of enzymatic and non-enzymatic antioxidants and in some cases, they even act as ROS scavengers (Ashkavand *et al*., 2018; Sun *et al*., 2021). Zaimenko et al. (2014) showed in their experiments that drought resistance of wheat and corn plants increases by applying analcite nanoparticles. Sun et al. (2021) observed increased photosynthesis, stomatal closure, increased relative amount of water and chlorophyll pigment in leaves, increased starch and sugar biosynthesis and increased glucose metabolism when ZnO nanoparticles were applied to drought-affected corn plants. When TiO_2 nanoparticles are applied to wheat plants grown in drought conditions, the intensity of photosynthesis, the percentage of germination, the formation of chlorophyll, the regulation of enzyme activity, the nitrogen metabolism and as a result, the growth of plants and the increase in productivity, Jaberzadeh et al. (2013) observed in their experiments.

It was determined that zero-valent iron nanoparticles maintain normal sensitivity to drought in Arabidopsis thaliana plants exposed to water stress, activate H-ATPase in the plasma membrane, cause stomata to open, increase chlorophyll content and biomass (Kim *et al*., 2015). It is known that plants are more sensitive to drought in the initial stage of development. Therefore, the application of nanoparticles at this stage allows us to see more clearly the effects of drought resistance in plants. When applying Fe, Cu, Co and ZnO nanoparticles in the initial vegetation period of soybean plant development, they found that the plants are easily adapted to drought. At this time, the morphology of sprouts and roots improves. These metal-based nanoparticles induce the expression of drought genes, resulting in increased plant resistance (Linh *et al*., 2020). In general, drought stress weakens the growth of plants and creates a physiological imbalance. However, experiments show that when Ag nanoparticles are applied, the morphological changes caused by drought stress improve in common beans even at cold temperatures. Photosynthetic pigments – Chl a, Chl b and carotenoids are very low in eggplant plants subjected to drought stress. However, when Ag nanoparticles are applied, the amount of these pigments increases (Alabdallah *et al*., 2021).

Thus, considering the role of nanotechnology in the problem of drought, the identification of nanoparticles that can improve the tolerance of plants to this important stress factor will lead to new approaches in agricultural practice. Research shows that some nanoparticles improve the drought resistance of plants, although the mechanism of action is not completely clear. One of these nanoparticles is zinc nanoparticles (ZnO) and interest in these nanoparticles has increased significantly in recent times. According to the results of experiments conducted in this field, ZnO nanoparticles have a positive effect on several biochemical and physiological processes in most plants grown in drought conditions. In addition, ZnO nanoparticles significantly increase the activity of antioxidant enzymes (SOD, GPO and to some extent PPO) in drought-stressed plants, increase the content of chlorophyll and protein, as well as POP activity. On the other hand, certain changes in the balance of P, Cu, Fe and Zn elements were observed in plants treated with ZnO nanoparticles. Considering the important role of ZnO nanoparticles in plant defense systems, it is relevant to investigate the effects of these nanoparticles depending on the type of plants and their developmental stages. These studies are of special interest from both theoretical and practical points of view.

2. Materials and Methods

The bean plant (Phaseolus vulgaris) Yalçın variety was used as an object in the experiments. Bean seeds were taken from the seed fund of the Institute of Genetic Resources of the Ministry of Science and Education of the Republic of Azerbaijan. The Yalcin variety was chosen because it is a drought-sensitive variety. The vegetation period is short and as it is well cultivated in laboratory conditions, it is useful as a model object in experiments. Bean seeds were selected, disinfected in H_2O_2 solution, dried and coated with ZnO nanoparticle powder. After 24 hours, seeds coated with ZnO nanoparticles were planted in vegetation pots and cultivated in Plant Growth Camber. In the normal version, the plants were watered with 100 ml of tap water every day at 9:00 a.m. local time. The drought stress variants were watered with 100 ml of water every 3 days. The composition of pigments was initially determined in samples taken from the leaves of 10-day-old plants. The measurement of the composition of pigments was continued until the bean formation period (40days).

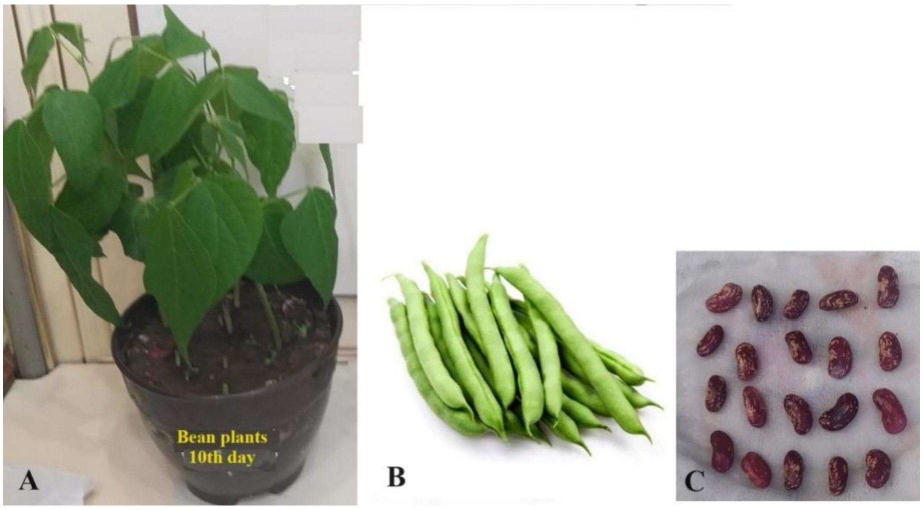

Figure 1. Yalchin bean variety: 10-day-old plant (A), pods (B) and seeds (C)

Nanoparticles. The nanoparticles ZnO (10-30 nm, purity 99,3%) and) in powder form were purchased from Sky Spring Nanomaterials, Inc (USA). The characteristics of the particles were as follows. Average particle size: 18 nm, purity: 99.9% and surface area > 80 (m^2/g) as reported by the commercial agent.

Methods for measuring pigments. The amount of chlorophylls and carotenoids was determined by a standard spectrometric method. For this, the fully expanded leaves were taken after 10 days of the seed sowing and the contents of chlorophyll a, b and carotenoids from the different trifoliate leaves. The samples were determined by extracting the leaves (0,1 g) in acetone (95%) in dark at 4°C and then the extract was centrifuged at 6000 cycles for 5 minutes, the amount of pigments was measured at wavelengths of 440, 645 and 663 nm using SPECORD 250 plus spectrophotometer. UV-vis spectroscopy is used to obtain absorption spectra of solids as well as substances in complex solutions. The electromagnetic spectrum of energy in UV-vis is 1.5-6.2 eV, which corresponds to wavelength values of 200-800 nm.

The amount of pigments was determined according to the following formulas:

Chl a (mg/g-fresh weight) = $9{,}784 x A_{663} – 0{,}990 x A_{645}$

Chl b (mg/g) = $21{,}426 x A_{645} – 4{,}650 x A_{663}$

Chl a +Chl b (mg/g) = $5{,}134 x A_{663} + 20{,}436 x A_{645}$

Carotenoids (mg/g) = $4{,}695 \cdot A_{440} – 0{,}288 \cdot (a + b)$

A - absorbency at corresponding wave length, values 9.784, 0.990, 21.426, 4.650 and 0.288 is the molar absorptivity coefficient according to Holm (1954) and Wetsttein (1957) for acetone (absorption of 1 cm). After calculating the concentrations, the amounts of pigment per g of fresh matter were calculated applying the formula:

$$C = \frac{c1Vr}{m}$$

C - content of pigment (mg/g) of fresh matter; $c1$- the concentration of pigment calculated by the previous formula (mg/l); V- the starting volume of extract (ml); r - dilution; m- the weighed fresh plant (g).

To determine the kinetics of changes in chlorophyll pigments using fluorescence spectra, bean leaves were used. At the 3rd trifoliate leaves of bean development, leaf samples 3 mm wide and 1 cm long were taken and after holding them for 1 hour in the dark, fluorescence spectra were recorded. Fluorescence spectra were recorded in a spectrophotometer (Cary Eclipse, Varian and Austrian). The Cary Eclipse is an optical instrument for measuring kinetic processes and allowing operation in fluorescence, phosphorescence, chemo- and bioluminescence modes. The device uses a xenon lamp as a source of illumination. The fluorometer can record the light rays emitted by the samples in 4 modes. The device's high scanning speed (24,000 mm/inch) allows you to acquire a full spectrum in 3 minutes. Excitation and emission spectra are in the range of 200-900 nm for leaves.

3. Results

The role of pigments, especially chlorophyll and carotenoids, is very important in green plants. Chlorophyll, as the main pigment of the photosynthesis process, is an irreplaceable biological molecule in the conversion of light energy into chemical energy and constituents. Other pigments involved in the photosynthesis process, carotenoids (red, orange or yellow pigments) also play an important role in the functional activity of the chlorophyll pigment. More than 600 carotenoids belonging to the class of phytonutrients are known in plant cells, bacteria and algae (Meléndez-Martínez *et al.*, 2014). Carotenoids absorb light from violet into the greenish-blue range. The set of wavelengths of light that pigments can absorb and activate is called their absorption spectrum. By determining the absorption spectra, it is possible to determine the structural and functional changes of pigments and their quantity. The three pigments that play an important role in absorbing sunlight in plants, chlorophyll a, chlorophyll b and beta-carotene, are very important in generating their responses to environmental factors. These pigments undergo serious changes in stressful conditions and as a result, they cause a decrease in the activity of the photosynthesis process, which is responsible for the productivity of plants. Therefore, by adjusting the functional and structural changes of these pigments under stressful conditions, it is possible to detect the adaptation and tolerance of plants to stress. In addition to detection, by influencing the synthesis and stability of these pigments with different classes of substances, it is possible to increase the resistance of plants to stresses. Nanomaterials also belong to this class of substances.

Bean seeds were planted in growth containers with normal soil and stable humidity and then placed in a Plant Growth Chamber (fitotron). Ten-day-old seedlings were fed regularly (once a week) with NPK fertilizer. Beans planted in 4 options (I normally irrigated, II droughts, III ZnO + normal irrigated, IV ZnO + drought) planted in Fitotron were grown for two weeks under optimal conditions and leaf samples were taken and the amount of pigments was determined as indicated in the methodology. In leaf samples, pigments (Chl a, Chl b, Chl (a+b) and carotenoids) were determined approximately every three days for 40 days. The leaf samples were selected according to the layers.

Figure 2. The general appearance of a 3-week-old bean plant grown in a Plant Growth Chamber: I – normally irrigated; II – drought; III – ZnO + normal irrigation; IV – ZnO + drought variant

Figures 2 and 3 show the general state of bean plant development during the 3-week and 7-week vegetation period, respectively. As can be seen from Figure 2, the rate of development is different in different variants in 3-week-old plants. A positive effect of ZnO nanoparticles on plant growth under normal irrigation conditions is felt. While in the other variants, there were 2nd layer leaves in the plants, in the 3rd variant ZnO nanoparticles accelerated the development of the plants and already the 3rd layer leaves were formed and creeping stems were formed in the plants. Development appears to be poor in drought-exposed plants. This is felt both in the II variant without nanoparticle application and in the IV variant with nanoparticle application. This tendency remains at each stage of plant development in all variants. As can be seen from Figure 3, the application of ZnO nanoparticles under normal irrigation conditions in 7-week-old plants (stage of bean formation) led to an increase in plant height and faster bean formation. It is interesting that in the drought variant, although ZnO nanoparticles did not change the height of the plants, they had a positive effect on the formation of beans.

The main goal of the experiments was to clarify the effect of drought stress on the synthesis of the main pigments (Chl a, Chl b and Carotenoids) during the 7-week vegetation period of the bean plant and the role of ZnO nanoparticles in combating this stress. The kinetics of the amount of pigments per unit wet weight in the leaf samples, which acts as an indicator of synthesis, was determined by the spectral method. Measurements were made 12 times during the 7-week vegetation period. Figure 4 shows the kinetics of changes in the amount of Chl a, Chl b and carotenoids in plants grown under normal irrigation conditions and subjected to drought stress. The amount of pigments was measured in different trifoliate leaf samples of the plants. Bean plants have trifoliate compound leaves. Each of the three leaflets is oval-shaped.

Figure 3. The general appearance of a 7-week-old bean plant grown in a Plant Growth Chamber:
I – normally irrigated; II – drought; III – ZnO + normal irrigation; IV – ZnO + drought variant

Measurement of pigments started from the first trifoliate leaves and continued until the 5th trifoliate leaves. It was clear that drought stress reduces the amount of Chl a pigment in the first, 3rd and 4th trifoliate leaves. But in other leaves, on the contrary, the amount of Chl a pigment increases due to drought. The amount of Chb pigment was higher in all the leaves taken for measurements under the effect of drought than in the irrigated plants. But only the 4th trifoliate was reduced in the leaf taken. From the kinetics of carotenoid changes, it was clear that drought stress does not seriously affect the synthesis of carotenoids, only a decrease is observed in the 3rd and 5th trifoliate leaves.

Figure 5 shows the kinetics of changes in pigment content in bean plants grown under normal irrigation conditions and treated with ZnO nanoparticles. Measurements were made on different leaves of bean plants for 7 weeks. The first measurement was taken from the 2nd trifoliate leaves. As can be seen from the Figure 5, the amount of Chl a was greater than the amount of Chl b and this trend was observed throughout the growing season. The diversity in the amount of Chl a was greater than that of the Chl b pigment. The amount of Chla was most observed in the 3rd trifoliate leaves. The amount of Chl b was also high in the 3rd trifoliate leaves. Increases and decreases in the amount of carotenoids were not observed. The amount of carotenoids remained almost the same in all leaves. Only in 5th trifoliate leaves, ZnO nanoparticles stimulated the synthesis of carotenoids.

Figure 6 shows the results of the effect of ZnO nanoparticles on the pigment content of the leaves of bean plants cultivated under normal conditions for 7 weeks. It was clear from the results of this experiment that the amount of Chla pigment varies depending on the condition of the leaves. It is maximum in the 3rd trifoliate leaves and then decreases slightly. Chl b pigment content is maximum in both 3rd trifoliate and 5th trifoliate leaves.

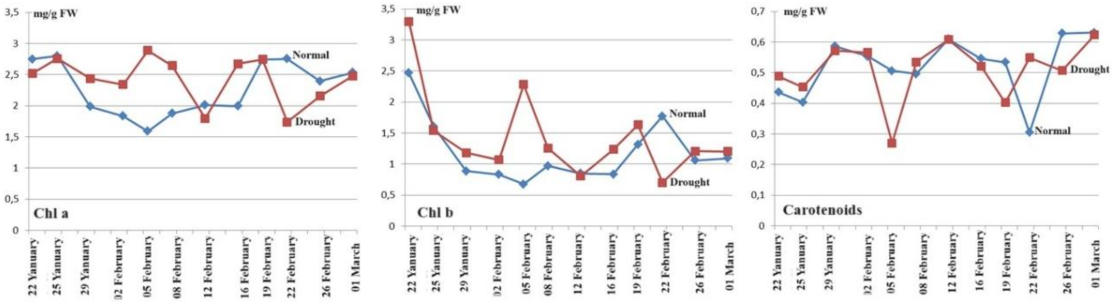

Figure 4. Kinetics of changes in the amount of Chl a, Chl b and Carotenoides during the 7-week vegetation period of bean plants cultivated under normal irrigation conditions

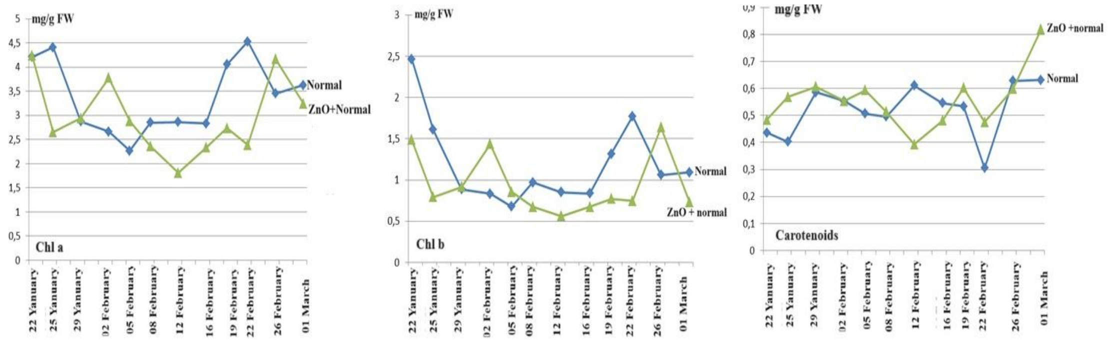

Figure 5. Kinetics of changes in Chl a, Chl b and Carotenoids during the 7-week vegetation period of bean plants treated with ZnO nanoparticles under normal irrigation conditions

The amount of carotenoids remains almost unchanged. Figure 7 shows the kinetics of pigment content changes in bean plants subjected to drought stress and treated with ZnO nanoparticles. From the results of these measurements, it was clear that the amount of Chl a in the 2nd trifoliate leaves of the plants grown under normal irrigation conditions and ZnO nanoparticles applied (1.5 mg/g FW) did not differ from the drought variant. However, the amount of Chl a in the 3rd trifoliate leaves was 3.4 mg/g FW in the irrigation variant, 2.95 mg/g FW in the drought variant and 2.35 mg/g FW in the drought variant where ZnO nanoparticles were not applied. ZnO nanoparticles also increased the amount of Chl b in drought conditions. ZnO nanoparticles had a positive effect on the amount of Chl b in other leaves in the drought variant. Thus, it can be said that ZnO nanoparticles have a positive effect on the pigment content of bean plants grown under drought conditions, depending on the condition of the leaves.

The kinetics of the change in the amount of Chl pigment under different cultivation conditions and with the application of ZnO nanoparticles was also studied by recording fluorescence spectra. For this, samples were taken from 3rd trifoliate leaves in all variants. Fluorescence spectra were recorded both in native leaves and in acetone extracts obtained from those leaves. As shown in the methodology, 3 mm wide and 1 cm long pieces were cut from the leaves and after keeping them in the dark for 1 hour, their spectra were recorded. An extract was prepared from the rest of the leaves in acetone and the fluorescence spectra of Chl were recorded. The results of these measurements are given in Figure 8.

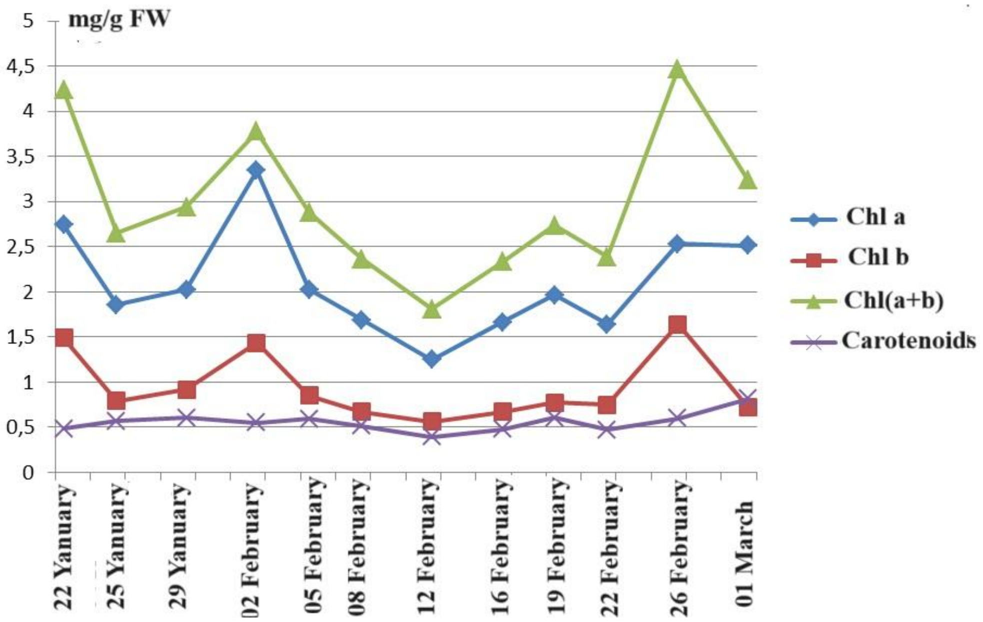

Figure 6. Kinetics of changes in pigments during the 7-week vegetation period of bean plants treated with ZnO nanoparticles under normal irrigation conditions

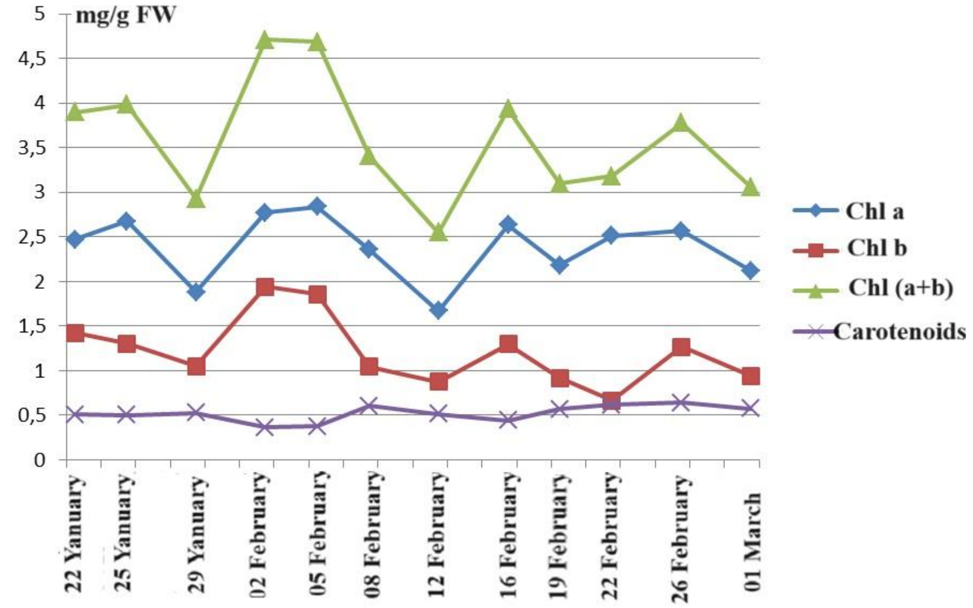

Figure 7. Kinetics of changes in pigments during the 7-week vegetation period of bean plants treated with ZnO nanoparticles under drought conditions

In these spectra, the fluorescence emission of Chl pigment was recorded in samples obtained from all variants. First, it should be noted that the fluorescence spectra recorded both in the leaves and in the extract are of the same nature. In both cases, the maxima of the fluorescence emission spectra of Chl coincide. In samples taken from different variants, the maximum of these spectra differs according to the intensity amplitude. It is clear from the spectra that the intensity of the fluorescence spectrum (spectrum 2) in plants grown under drought conditions is higher than in other variants. The maximum intensity in the fluorescence spectrum was further increased when ZnO nanoparticles were applied under drought conditions. Through these spectra, it is possible to determine the amount of Chl. To calculate this, it is necessary to take the ratio of the value of the spectrum intensity at the wavelength of 650.00 to the value at the wavelength of 709.01 (F650/709). In plants cultivated under normal irrigation conditions, this value will be F650/709 = 65.5/34.8 = 1.9 mg/g.

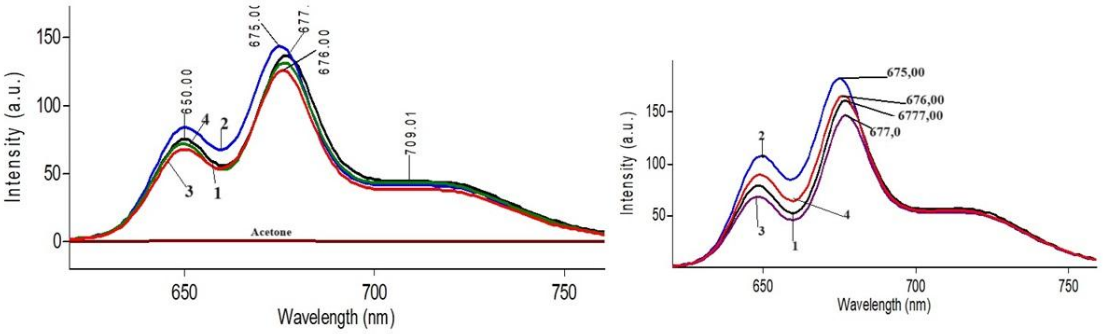

Figure 8. Fluorescence spectra of Chl pigment of 3rd trifoliate leaves of bean plant in acetone extract of (left) and native leaves (right): 1 – normally irrigated; 2 – drought condition; 3 – ZnO + normal irrigation; 4 – ZnO + drought variant

Since the effect of nanoparticles on the degradation of chlorophyll and pigments is also interesting, the effect of ZnO nanoparticles on the degradation of pigments was investigated in our experiments with beans. Chlorophyll degradation is a natural process, but as long as its balance is maintained, it can be altered by several environmental factors. It is known that green leaves turn yellow both naturally and under the influence of certain factors. This process occurs as a result of the degradation of chlorophyll pigment. The decomposition of chlorophyll is strongly dependent on the light factor. Long-term Experiments have shown that when leaves are kept in complete darkness at normal temperature (30°C) for a long time, chlorophyll degradation occurs quickly, within 3 days. Very little white light with a photon intensity of 0.5 µmol m-2.s-1 significantly slows the breakdown of chlorophyll. Red or far-red light has little effect on chlorophyll degradation in the dark. It has been studied that the breakdown of chlorophyll is regulated by phytochrome. It has also become clear that the breakdown of chlorophyll is related to the degradation of the membrane proteins that transport it (Katsuhiko *et al*., 1992). The effect of nanoparticles on the photosynthesis process, as well as on the pigment composition, has been studied in detail. However, there are very few experimental facts about the effect of nanoparticles on the degradation of chlorophyll (Ahmadov & Hasanova, 2023). It is important to study this issue from a practical point of view. Regulation of chlorophyll degradation is an important issue in agricultural practice. Therefore, in our experiments, we examined the effect of ZnO oxide nanoparticles on chlorophyll degradation in bean leaves grown under normal irrigation conditions and subjected to drought stress.

For this, we took 3rd trifoliate leaf samples from all variants of beans that we used in our experiments and kept them in complete darkness for 10 days. An illustration of the leaves stored in the dark is given in Figure 9. It was found that leaves taken from all variants turned yellow, that is, chlorophyll degradation occurred, but ZnO nanoparticles were applied and chlorophyll degradation did not occur in the leaves of bean plants subjected to drought stress. Within 10 days, the leaf remained completely green.

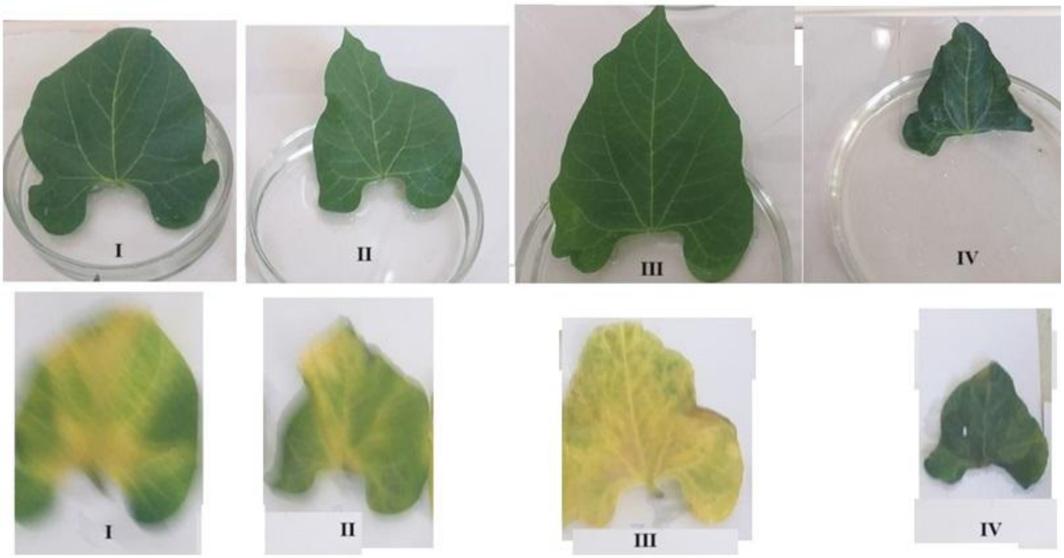

Figure 9. Chl pigment degradation of 3rd trifoliate leaves of bean plant: I – normally irrigated; II – drought condition; III – ZnO + normal irrigation; IV – ZnO + drought variant.

4. Discussion

Zinc is a useful element in the growth and development of plants. Zinc maintains cell turgor, regulates cell homeostasis and improves cell structure and anatomical features in stressed plants (Rizwan *et al*., 2019a). Taking these into account, in the research presented, using the nanoparticle form of zinc, that is, ZnO nanoparticles, an attempt was made to obtain results that would contribute to the improvement of drought resistance in bean plants. Zinc plays an important role in plants, especially in cereals such as wheat and its high accumulation is very important in reducing abiotic and biotic stresses. Experiments have shown that the amount of zinc element in plants can be increased by applying ZnO nanoparticles (Dimkpa *et al*., 2017b; Taran *et al*., 2017; Ali *et al*., 2019). Zinc accumulation leads to the minimization of ROS in leaves. As we mentioned above, the mechanism by which nanoparticles are involved in drought tolerance responses in plants is complex. However, there are many different nanoparticles that can improve the drought tolerance of plants by altering their morphophysiological, biochemical and even gene expression.

The results of our experiments have shown that ZnO nanoparticles can play a role in the drought resistance of bean plants. It is clear from the results that ZnO nanoparticles, when applied under normal irrigation conditions, have a positive effect on the height development of plants, reduce the vegetation period and keep the pigment content stable. The difference in the pigment content observed in our experiments during the growing season may be related to the formation of bean leaves in different layers at different times. It is interesting that in the plants subjected to drought stress (variant IV) where ZnO nanoparticles were applied, the pigment content remains stable, although the growth of the plants is not fast., the growing season is shortened and pod formation occurs more quickly. In addition, Chl pigment degradation does not occur for a long time, the leaves retain their greenness.

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