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CONTENT

CHLOROPHYLL DEGRADATION IN LEAVES OF NANOPARTICLES EXPOSED COTTON SEEDLINGS UNDER DARK CONDITION

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Abstract. *The aim of this study was to determine the effect of nanoparticles on the aging of cotton leaves by measuring the content of chlorophyll. Until now, the mechanism of chlorophyll degradation and the factors influencing this process have not been fully studied. In the presented research, the effect of nanoparticles on the degradation of chlorophyll and carotenoids, photosynthetic rate, Fv/Fm ratio and production of oxidants, ie. H2O2 and superoxide radical (O2-), lipoxygenase enzyme activity (LOX), and antioxidant enzymes SOD and CAT activity in 14-day-old cotton seedlings under dark conditions was investigated. Metal-based nanoparticles (ZnO, CuO, Fe2O3,TiO2) were used in the experiments. Cotton was grown in a controlled environment plant growth chamber. First, the compounds of chlorophyll and carotenoids were measured in the leaves of the third tiers of 14-day-old cotton seedlings, and then the seedlings were kept in the dark. The kinetics of the process depends on the composition and size of the nanoparticles. Nanoparticles do not significantly affect the increase of H2O2, superoxide anion during chlorophyll degradation, but ZnO nanoparticles do. Lipoxygenase, SOD and CAT enzymes activity in this process of nanoparticles is not seriously affected.*

Keywords: *nanoparticles, chlorophyll degradation, qhydrogen peroxide, superoxide anion, enzyme activity.*

INTRODUCTION

Yellowing of leaves and ripening of fruits is a natural process in plants, and degradation of chlorophyll occurs at this time. Studying the mechanism of synthesis and degradation of chlorophyll, which is the main pigment of the photosynthesis process in green plants, is very important from both a scientific and a practical point of view. According of the modern scientific literature synthesis of chlorophyll has been widely studied. The synthesis mechanism of chlorophyll and the stages of this process, influencing and regulating factors have been studied in detail. However, it is known that along with the synthesis of chlorophyll, its degradation also occurs. Degradation of chlorophyll occurs both during the development of plants and when they complete their ontogeny. Synthesis of chlorophyll in perennial plants (for example, woody plants) occurs during the autumn season in connection with the yellowing of leaves and the ripening of fruits. Therefore, the process of chlorophyll degradation can be considered as a natural process.

Degradation of chlorophylls in leaves occurs mainly through pheophorbide oxygenase

(PAO). Recently, 6 types of enzymes responsible for chlorophyll catabolism have been identified and their functions have been clarified. Chlorophyll catabolic enzymes (CCEs) are: chlorophyll b reductase (Horie et all., 2009), 7-hydroxymethyl chlorophyll a reductase [Meguro et all., 2011], Mg2+-dechelatase [Shimoda et all., 2016], pheophytinase [Shelbert et all., 2009] pheophorbide a oxygenase [Pružinská et all., 2003], and reductase of red chlorophyll catabolite, RCC [Pružinská et all., 2007]. During the degradation of chlorophyll, the final and intermediate products and their structures, as well as the biochemistry of the porphyrin decomposition reaction, have been elucidated. It has been established that the intracellular localization of the catabolic pathway is particularly important in the regulation of chlorophyll degradation [Matile et all., 1999].

However, it is also known that various biotic and abiotic factors affect the degradation of chlorophyll at all stages of plant development. The main environmental factors affecting leaf yellowing (senescence) in all plants, including cotton, are light, temperature, humidity, drought, salinity, and mineral nutrition. For example, light is not only the main energy source of the photosynthesis process, but also an important regulatory factor for the synthesis and degradation of chlorophyll. In photosynthesizing tissues, light also ensures the formation of auxin hormone. The period of illumination plays an important role in the regulation of growth and development, aging of plants [Wu et all., 2012b]. Light intensity and finally darkness plays an important role in chlorophyll degradation and premature yellowing of leaves in cotton plant [Chen et all., 2016]. In most cases, red spots appear on the leaves of the cotton plant. The reason for the red coloring of the leaves is the effect of various abiotic stress factors. At this time, chlorophyll is degraded in the leaves, anthocyanins, proline accumulate rapidly and the activity of peroxidase increases. This is a coordinated measure to overcome abiotic stress in the cotton plant [Edreva et all., 2002].

These are the main factors that affect the degradation of chlorophyll. It is known that at low temperatures [Taylor & Graig, 1971], in excessively high and continued hot weather [Adelisi & Lawanson, 1978]. Degradation of chlorophyll occurs rapidly during ultraviolet and gamma radiation. Edaphic stresses, such as nitrogen deficiency [Ericsson et all., 1982], as well as iron deficiency [Sprague, 1964], have an important effect on the decomposition of chlorophyll.

Experiments have shown that the lack of potassium element, which plays an important role in mineral nutrition of plants, increases the rate of early season flowering and yellowing of leaves in cotton plants and reduces the number of leaves, leaf area, number of stems, weight of cotton seed per boll and percentage of lint [Hu et all., 2016].

The application of nanotechnology in agriculture is considered one of the important approaches to increase crop production, and this approach has good prospects for the future. Therefore, the main way to increase plant productivity using nanotechnology is to study the molecular mechanism of the action of its materials, especially nanoparticles, on plant development, their habitat, and important physiological and biochemical processes. It is known that the process of photosynthesis plays an important role in plant productivity, and this process is based on the pigment chlorophyll. The amount of chlorophyll pigment, its function and lifespan are particularly affected by environmental factors, as well as the natural aging of the leaves. At this time, you can regulate the activity of photosynthesis by observing the process of synthesis and degradation of chlorophyll. In the presented article, an attempt was made to develop the primary mechanism of action of nanoparticles on chlorophyll degradation and methods to control it.

MATERIALS AND METHODS

Ganja-110 cotton variety was used in the experiments. This variety of cotton has been regionalized in Azerbaijan since 2009. Obtained

by experimental mutagenesis. The seeds of Ganja-110 cotton variety were presented by the Institute of Genetic Resources of ANAS. Cotton gins are cleaned of fibers before sowing. This variety of cotton is fast-growing, the height of the plant is 90-110 cm, the bush is compact, pyramidal in shape, monopodial branches - 1, the stem is pale, green, weakly hairy, resistant to dormancy. The leaf is medium-large, 3-5-lobed, finger-shaped, dark green, heart-shaped, the flower is medium-large, yellow cream color, no anthocyanin spot, the pollen is yellow. The cone is large ovoid, star-shaped, smooth, brown-spotted, green in color. The product does not spill. The seed is medium large, 1000 seeds weigh 115-120 g, egg-shaped, dark green, moderately hairy [Humbatov & Khalilov, 2012].

Nanoparticles.The nanoparticles (Fe2O3, CuO, ZnO and TiO_2) 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 (m2/g) as reported by the commercial agent. Maize seeds were dusted with nanoparticle powder before sowing. For this, 1 mg of each nanoparticle was added to a 50 ml glass for 10 seeds, and the seeds were processed in a shaker for 10 minutes.

Methods for measuring pigments. The amount of chlorophylls and carotenoids was determined by a standard spectrometric method. For this, 0.1 g of leaves are crushed in a mortar and 10 ml of 95% acetone is added and mixed. After the extract was centrifuged at 6000 cycles for 5 minutes, the amount of pigments was measured at wavelengths of 440, 645, 663 nm using SPECORD 250 plus spectrophotometer. The amount of pigments was determined according to the following formulas:

Chl a (mg/g-fresh weight) = 9,784xA663 – 0,990xA645

Chl b (mg/g) = 21,426xA645 – 4,650xA663

Chl a +Chl b (mg/g) = 5,134xA663 + 20,436xA645

Carotenoids (mg/g) = 4,695·A440 – 0,288·(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 - 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).

In addition to variation in pigment composition, photosynthetic rate, Fv/Fm ratio and production of oxidants, ie. H_2O_2 and superoxide radical (O2-), lipoxygenase enzyme activity (LOX), and antioxidant enzymes SOD and CAT activity is set. Three replicates were performed to measure total pigment content, Fv/Fm ratio, oxidant production, antioxidant enzyme assays. The maximal efficiency of PS II photochemistry (Fv/Fm) in the dark-adapted state was calculated (Fv/Fm = (Fm) Fo)/Fm), using both light and dark fluorescence parameters. The rate of photosynthesis was measured at photosynthetic photon flux >600 lE m)2 s)1 (saturating light), using an exceptional sensitivity and reproducibility the Photosynthesis Yield analyzer MINI-PAM (Germany).

For the meusering the H2O2 level was used colorimetrically method as described by Okuda et all. [1991] and expressed in nm g-1 FW. Superoxide anion was estimated according to Chaitanya and Naithani [1994] and expressed as change in OD min-1 g-1 FW. The lipid peroxidation was determined according Behra et all. [1999] by MDA content produced by thiobarbituric acid reaction at low pH and expressed as MDA content in µg-1 fresh weight (FW). Lipoxygenase was measured spectrophotometrically at 234 nm [Gallego et all. 1996] and expressed as µm min-1 mg-1 protein. MSI was determined by recording the electrical conductivity of leachates in double distilled water at 40 and 1000C [Chaudhuri & Choudhuri, 1992] and expressed as percentage

For assays of activity SOD and CAT enzymes , frozen tissue was homogenized in ice-cold 0.1 m Tris–HCl buffer at pH 7.8 containing 1mm EDTA, 1 mm dithiothreitol and 5 ml of 4 % polyvinyl pyrrolidone g-1 FW. The homogenate was filtered through a nylon mesh and centrifuged at 20 000 g at 40C. The supernatant was used for measuring enzyme activity. Superoxide dismutase (SOD) activity was determined by nitroblue tetrazolium (NBT) method by measuring the photoreduction of NBT at 560 nm (Beyer and Fridovich, 1987). One unit of SOD activity is equivalent to the amount required to inhibit photoreduction of NBT by 50 %. Catalase activity (CAT) was estimated according to Samantary (2002) and expressed as µm H_2O_2 destroyed min-1 mg-1 protein.

RESULTS AND DİSCUSSİON

The local Ganja-110 cotton variety, which is the most widely used in both field and laboratory studies, was used as an experimental object. Germination of cotton seeds is almost one hundred percent under normal conditions. In order to obtain cotton seedlings, 5 seeds per pot were planted in vegetation pots and cultivated in the Plant Growth Chamber, phytotron. The humidity in the phytotron was 70%, the temperature was 250C, and the illumination was 20,103 lk. When the sprouts are 14 days old, the third leaf has already started to form. Samples were taken from the first, second and third leaves of the seedlings to measure the amount of chlorophyll and carotenoids, as well as other biochemical and physiological parameters. Initially, the amount of chlorophyll a and b, chlorophyll (a+b) and carotenoids was measured according to the procedure given in the methodology (fig. 1). After determining the content of the pigment, the lighting in the phytotron was stopped and the seedlings were kept in complete darkness for 9 days.

The amount of variation in pigment composition in normal seedling leaves and exposed on nanoparticles in dark condition is given figure 2. The control variant represents measurements of the amount of pigments before placing the plants in the dark, under normal conditions, after keeping them in the dark for 168 hours and 216 hours. The analysis of the pigment composition showed that the amount of chlorophyll a in the leaves of 14-day-old bean sprouts was 1.75 mg/g FW, chlorophyll b 0.6 mg/g FW, chlorophyll (a+b) 2.5 mg/g FW, and the amount of carotenoids was 0.47 mg/g FW. When the sprouts are left in the dark for 7 and 9 days in normal water without nanoparticles, the amount of chlorophyll and carotenoids decreases sharply (fig. 2, control option). However, when the sprouts are kept in dark conditions in water with nanoparticles for 7 and 9 days, the pigment content changes in different ways. This change depends on the type of nanoparticles. Thus, the amount of both chlorophyll and carotenoids in TiO_2 nanoparticle does not change drastically. This indicates that TiO_2 prevents the degradation of pigments. Other nanoparticles (CuO, ZnO, $Fe2O_3$) slow down the degradation of pigments (fig. 2).

Figure 1. *The 14 days-old seedlings of cotton plant.*

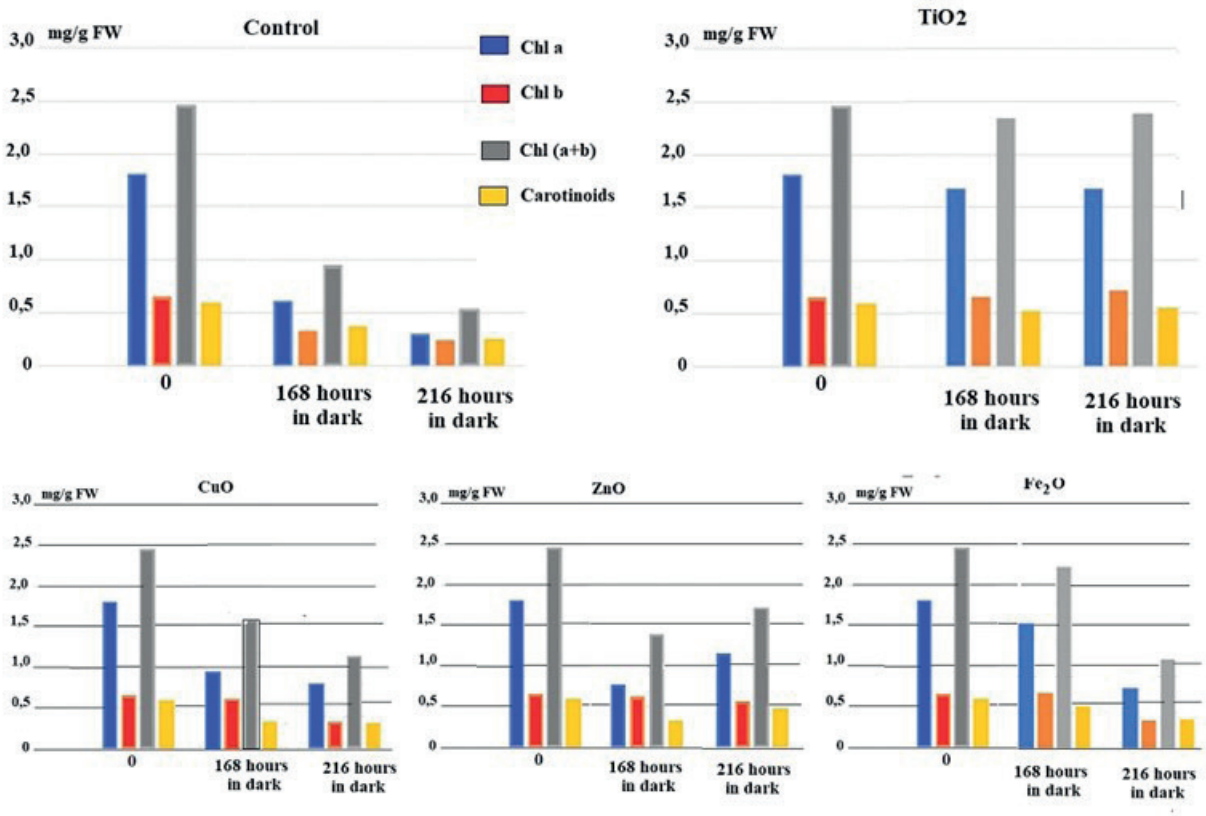

Figure 2. *The amount of variation in pigment composition in normal seedling leaves and exposed on nanoparticles in dark condition.*

The Fv/Fm ratio decreased significantly in both control and nanoparticles exposed plants during degradation of chlorophyll in case of dark condition. Fv/Fm ratio in the control variant decreased from 0.87 ± 0.023 to 0.32 ± 0.013 after 9 days of darkness. Fv/Fm ratio in the control variant decreased from 0.87 ± 0.023 to 0.32 ± 0.013 after 9 days of darkness. However, the amount of Fv/Fm ratio in the sprouts stored in nanoparticles did not decrease dramatically. The most effective of these nanoparticles was the TiO_2 nanoparticle. Due to its effect, the amount of Fv/Fm ratio decreased from 0.87 ± 0.023 to 0.67 ± 0.017 within 9 days. The greatest reduction was caused by CuO nanoparticles. The results of these measurements are given in Table1.

Fv/Fm ratio					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	0.87 ± 0.023	0.87 ± 0.023	0.87 ± 0.023	0.87 ± 0.023	0.87 ± 0.023
168 hours	0.44 ± 0.016	0.72 ± 0.014	0.53 ± 0.012	0.68 ± 0.019	067 ± 0.013
216 hours	0.32 ± 0.013	0.67 ± 0.017	0.37 ± 0.018	0.47 ± 0.011	0.44 ± 0.015

The concentration of H_2O_2 increased significantly from 6.9 ± 0.51 to 20.4 ± 0.32 in control plants after 9 days of darkness. The amount of H_2O_2 concentration in the sprouts stored in nanoparticles did not increase dramatically. The greatest increasing was caused by TiO_2 and ZnO nanoparticles. The results of these measurements are given in Table 2.

Table 2.

Changes in hydrogen peroxide concentration in leaves of control and exposed on nanoparticles in dark condition

Hydrogen peroxide (H2O2) concentration (nm g-1 FW)					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	6.9 ± 0.51	6.9 ± 0.51	6.9 ± 0.51	6.9 ± 0.51	6.9 ± 0.51
168 hours	11.8 ± 0.41	13.7± 0.61	15.8 ± 0.33	14.9 ± 0.51	12.9 ± 0.63
216 hours	20.4 ± 0.32	19.6 ± 0.31	17.9 ± 0.42	19.8 ± 0.23	18.9 ± 0.23

The concentration of superoxide anion (O_2-) increased significantly from 1.09 ± 0.12 to 2.19 ± 0.17in control plants after 9 days of darkness. The amount of superoxide anion (O_2-) concentration in the sprouts stored in nanoparticles did not increase dramatically. The greatest increasing was caused by ZnO nanoparticles. The results of these measurements are given in Table 3.

Table 3.

Changes in superoxide anion concentration in leaves of control and exposed on nanoparticles in dark condition

Superoxide anion (O_2-) concentration (Δ OD min-1 g-1 FW)					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	1.09 ± 0.12	1.09 ± 0.12	1.09 ± 0.12	1.09 ± 0.12	1.09 ± 0.12
168 hours	1.57 ± 0.22	1.97 ± 0.28	1.77 ± 0.42	2.07 ± 0.32	1.87 ± 0.43
216 hours	2.19 ± 0.17	2.39 ± 0.38	2.59 ± 0.26	3.09 ± 0.37	2.59 ± 0.33

Lipoxygenase (LOX) enzyme activity of cotton leaf was 7.1 ± 0.63 µm min-1 mg-1 protein in control plants. However, in the cotton plants sprouts stored in nanoparticles the activity of LOX enzyme was dependence of types of nanoparticles from 22.1 ± 0.54 to 23.3 ± 0.44 after 9 days of darkness. The LOX enzyme activity showed significant difference during deqradation of piqments. It is revealed that maximum membrane injury was seen in controls compared with nanoparticles exposed plants. The results of these measurements are given in Table 4.

Table 4.

Changes in lipoxygenase enzyme activity in leaves of control and exposed on nanoparticles in dark condition

Lipoxygenase (LOX) enzyme activity (µm min-1 mg-1 protein)					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	7.1 ± 0.63	7.1 ± 0.63	7.1 ± 0.63	7.1 ± 0.63	7.1 ± 0.63
168 hours	17.3 ± 0.58	15.2 ± 0.47	14.6 ± 0.43	18.3 ± 0.67	19.1 ± 0.51
216 hours	25.2 ± 0.61	22.1 ± 0.54	19.4 ± 0.64	23.3 ± 0.44	21.3 ± 0.14

It was observed that both of the antioxidant enzymes activity superoxide dismutase (SOD) and catalase (CAT) was higher in control cotton plants sprouts stored in nanoparticles after 9 days of darkness. Significant increase in the activity of antioxidant enzymes, SOD and CAT was seen in leaves stored in ZnO nanoparticles amount of 29.19 ± 1.8 and 30.12 ± 1.7 respectivle. Increase in SOD activity was observed from 15.59 ± 1.5 in control as against a much higher increase of 29.19 ± 1.8 enzyme unit mg-1 protein in stored in ZnO nanoparticles after 9 days of darkness plants, also a much higher increase of 30.12 ± 1.7 enzyme unit mg-1 protein showed in CAT activity in this particles (Table 5 and 6).

Table 5.

Changes in superoxide dismutase (SOD) enzyme activity in leaves of control and exposed on nanoparticles in dark condition

Superoxide dismutase (SOD) enzyme activity (enzyme units mg-1 protein)					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	15.59 ± 1.5	15.59 ± 1.5	15.59 ± 1.5	15.59 ± 1.5	15.59 ± 1.5
168 hours	19.29 ± 1.7	20.28 ± 1.3	21.47 ± 1.2	27.12 ± 1.6	22.89 ± 1.5
216 hours	25.34± 1.9	28.33 ± 1.6	27.39 ± 1.3	29.19 ± 1.8	26.11 ± 1.7

Table 5.

Changes in catalase (CAT) enzyme activity in leaves of control and exposed on nanoparticles in dark condition

Catalase (CAT) enzyme activity (µm H_2O_2 destroyed min-1 mg-1 protein)					
Exposition in dark	**Control**	**TiO_2**	**CuO**	**ZnO**	**Fe_2O_3**
O (hour)	18.52 ± 1.8	18.52 ± 1.8	18.52 ± 1.8	18.52 ± 1.8	18.52 ± 1.8
168 hours	22.31 ± 1.7	23.12 ± 1.6	26.41 ± 1.9	27.32 ± 1.8	20.22 ± 1.7
216 hours	25.42 ± 1.1	28.32 ± 1.3	28.36 ± 1.5	30.12 ± 1.7	29.72 ± 1.9

DISCUSSION

Natural leaf senescence is the last developmental stage in annual plants between the end of ontogenesis and their death. At this stage, first of all, chlorophyll, as well as some pigments, are degraded and transformed into others. In particular, the degradation of chlorophyll is characterized by a decrease in photosynthesis. This catabolic process causes plants to drop their leaves. Early shedding of leaves in the cotton plant is an important condition for the ripening of the cones and quality harvesting (especially by mechanical method). Therefore, the control of the synthesis and degradation of the chlorophyll pigment, which causes yellowing and reddening of the leaves in the cotton plant, is of great importance from a practical point of view. Experiments show that the degradation of chlorophyll can occur not only naturally, but also under the influence of various factors. It was determined that during the degradation of chlorophyll, the synthesis of active forms of oxygen (ROS), superoxide anion (O2), hydroxyl radical, and H2O2 remains uncontrolled and their amount increases. Degradation of chlorophyll is observed with a decrease in the rate of photosynthesis, an increase in various oxidants, antioxidant enzyme activity and an increase in the amount of antioxidants. Studies with nanoparticles have shown that they can prevent the degradation of chlorophyll.

The results of our experiments confirm this idea. So, if normally the degradation of chlorophyll occurs rapidly in the dark, this process is slowed down by the effect of nanoparticles. The kinetics of the process depends on the composition and size of the nanoparticles. Nanoparticles do not significantly affect the increase of H2O2, superoxide anion during chlorophyll degradation, but ZnO nanoparticles do. Lipoxygenase, SOD and CAT enzymes activity in this process of nanoparticles is not seriously affected. Thus, it can be concluded that nanoparticles can play an important role in the regulation of chlorophyll degradation.

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