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Effect of the $[CuL_2]Br_2$ complex on photosystem II

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Modern agriculture relies on the use of genetically modified crops that can withstand the effects of herbicides used to kill weeds. However, despite advances in biotechnology, there remains and even increases the need to develop new, sustainable, environmentally friendly, and biodegradable herbicides. Photosynthesis is an attractive target for inhibitory compounds, both for the creation of new means to control weeds and the study of photosynthetic reactions. In this work, the effect of a new synthesized complex of copper(II) bromide $[CuL_2]Br_2$ (where L = bis{4H-1,3,5-triazino[2,1-b]benzothiazole-2-amine,4-(2-imidazole)}) on the functional activity of photosystem II (PS II) membranes isolated from spinach. The absence of photosynthetic oxygen evolution in PS II membranes in the absence of artificial electron acceptors, but in the presence of the $[CuL_2]Br_2$ complex, showed that this compound is not capable of acting as an artificial electron acceptor for PS II. When artificial electron acceptors were added, the photosynthetic oxygen evolution reaction was inhibited by $[CuL_2]Br_2$ complex. The complex also caused suppression of photoinduced changes in variable chlorophyll (F_V) fluorescence associated with photoreduction of the primary quinone acceptor Q_A. The inhibition of both major PS II processes depended on the concentration of $[CuL_2]Br_2$. In all the concentrations studied, the decrease in F_M level occurred solely due to a decrease in F_V, while the value of F_0 remained stable, and no slowdown in photoinduced F_M growth was observed. Besides it, $[CuL_2]Br_2$ doesn't have any absorption bands either in the wavelength range corresponding to the actinic and measuring light, or in the emission region of chlorophyll fluorescence.

Keywords: *Photosynthetic inhibitors, copper, organometallic complex, photosystem II, oxygen evolution, fluorescence yield*

INTRODUCTION

Herbicide-resistant weed species that are constantly appearing, especially fast-growing ones, are one of the main factors limiting the yield of economically important crops. Weeds are still a limiting factor in the cultivation of crops around the world. Weed growth control mainly depends on herbicides, as they have high efficiency and relatively low cost compared to other control methods. Advanced technologies for increasing yields by creating genetically modified plant species also include the simultaneous use of herbicides to kill weeds (Ahmad & Mukhtar, 2017). Resistance to these herbicides in cultivated plants was created by genetic modification. However, even with such methods, it is necessary to search for more effective inhibitors that will be able to act at the lowest possible concentrations and be able to selectively suppress photosynthetic reactions in plant cells, which will make them safer for humans and fauna (Pospíšil, 2009). Searching among existing agents or creating new chemical compounds, as well as screening them

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for possible inhibitory properties in relation to various key reactions of the plant organism, can be the first step towards creating new herbicides and crop protection products (Gonec et., 2017). This becomes especially important, taking into account the growing population of the Earth. Most of the known herbicides effect on photosynthesis of plants (Schütte et al., 2017). The use of herbicides is still the most effective method of controlling the growth of weeds. However, plants continue to develop resistance to widely used herbicides, which is a major challenge for agriculture. In addition, frequent use of the same compounds causes contamination of water and soil, and the substances themselves can harm other living organisms (Trebst, 2007). Thus, in order to reduce the harm caused to the environment, special attention should be paid on new effective selective compounds acting by different mechanisms.

Photosynthesis is a complicated chemical process of converting the energy of sunlight into the energy of chemical bonds. As a vital process for all photosynthetic organisms, photosynthesis is an attractive target for the action of inhibitory substances (Vass, 2012). Inhibitory compounds can be used as a tool to study the mechanism of photosynthetic reactions. There are many examples in the literature of the use of various compounds to block individual sections of the electron transport chain in order to study in detail the processes of electron transfer. Nowadays, there are many substances capable of inhibiting key reactions of photosynthesis and important enzymes of the plant organism to varying degrees.

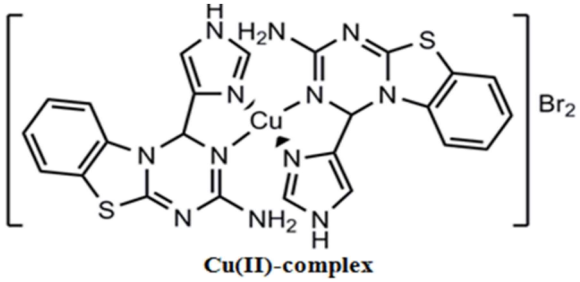

Fig. 1. Cu(II)-complex (Bis{4H-1,3,5-triazino [2,1-b]benzothiazole-2-amine,4-(2-imidazole)} copper (II) bromide).

However, compounds that act only on one of the metabolic pathways of a plant are not so effective due to the evolving resistance of plants to their action (Kilpin & Dyson, 2013).

Therefore, the creation of a universal inhibitor that is able to suppress a whole range of vital reactions is a rather promising approach for solving this problem.

Copper is an essential element involved in a variety of metabolic processes in plants, algae, and cyanobacteria. Despite its key role as a cofactor of a number of enzymes, free Cu ions are capable of causing oxidative damage to cells through the generation of reactive oxygen species (Karki, et., 2000). Although copper is essential for the normal growth and development of plants, excess Cu(II) ions exhibit the greatest toxicity among heavy metals. Studies have shown that the components of photosystem II (PSII) are more vulnerable to the inhibitory effects of copper compared to the elements of photosystem I (PSI). It is assumed that Cu can affect both the donor and acceptor sides of PSII. There is evidence that excess copper reduces the activity of the PSII reaction center, promotes the degradation of chlorophyll, and suppresses the function of the water-oxidative complex (Kilpin & Dyson, 2013). In addition, excess Cu can disrupt photochemical processes in PSII and cause structural changes in thylakoid membranes, which generally reduces the efficiency of photosynthetic systems. In this work, the effect of newly synthesized Cu(II) complex on the photochemical activity of PSII in plants was investigated.

MATERIALS AND METHODS

Research objects: PSII preparations isolated from the leaves of two-week-old garden spinach (*Spinacia oleracea*) plants and were used as objects of research. New organometallic complex based on divalent copper Cu(II)-complex (Bis{4H-1,3,5-triazino [2,1-b]benzothiazole-2-amine,4-(2-imidazole)}copper(II) bromide) (Fig.1) was synthesized at Gazi University, Ankara, Turkey. The copper(II) complex was synthesized by the electrochemical method described in (Tuck, 1979), using a platinum cathode (1x1 cm) and a copper plate as a protective anode.

Thylakoid membranes from spinach (*Spinacia oleracea*) were isolated in accordance with the method described earlier (Berthold et., 1981; Ford & Evans 1983; Klimov et., 1982). Two-week-old spinach leaves were thoroughly washed with distilled water and left overnight in a cold room (4°C) for starch disposal, after removing the central vein. Further, all operations were performed at a temperature of no more than 4°C in a weak green light. All buffer solutions were pre-cooled to 4°C. The leaves were placed in a laboratory blender and added isotonic buffer A (400 mm sucrose; 20mM HEPES (NaOH), pH 7.8; 15mM NaCl; 1mM EDTA; 1 mg/ml sodium ascorbate). The leaves were homogenized for 15 seconds and allowed to stand for at least 10 minutes. The resulting homogenate was filtered through 4 layers of special cloth with a minimum pore size of no more than 20 microns into a container placed on ice. Next, the filtrate was centrifuged for 5 min at 1000g (4 S) on a K-70 centrifuge (Universal 32R, Hettich Zentrifugen, Germany) to remove in homogenized tissue residues. The resulting supernatant was selected and centrifuged for 30 min at 10,000 g (4 S) to precipitate chloroplasts. The chloroplast precipitate was resuspended in hypotonic buffer B (20 mM HEPES (NaOH), pH 7.8; 15 mM NaCl; 10 mM $MgCl_2$).

The resuspended chloroplasts in buffer B were incubated on ice for 10 minutes to allow them to burst. After that, the suspension was centrifuged for 30 min at 10,000 g (4 S). Thylakoid membranes were obtained in the sediment, which were resuspended with 10% glycerin and stored at -80 C for further experiments. Native subchloroplast membrane particles enriched with PSII (BBY particles) were isolated from spinach according to the procedure. Spinach thylakoids in a primary concentration of 3 mg/ml were pre-washed from glycerol and centrifuged for 30 min at 5000g. The washed thylakoids were resuspended in buffer B. The suspension was incubated in the dark on ice for 1 hour, stirring constantly to ensure a more complete separation of PSII from PSI. 40 minutes after the start of incubation, chlorophyll was determined to calculate the required amount of detergent (20% Triton X-100: chlorophyll in a ratio of 25:1). The detergent of the desired concentration was diluted in buffer B and added to the suspension of thylakoids slowly over a glass rod with gentle continuous stirring on a magnetic stirrer, with the final chlorophyll concentration being 2 mg/ml. The mixture was incubated on ice for 20 minutes, stirring constantly. To remove possible starch grains and insolubilized material, the suspension was centrifuged for 5 min at 5000g (Beckman Coulter Avanti centrifuge, Beckman Coulter Inc., USA, rotor JA-18). After that, the supernatant was centrifuged for 30 min at 40,000 g (4 S). The precipitate containing BBY particles was resuspended in buffer B and centrifuged under the same conditions. It was repeated at least 3 times to wash off the detergent until the supernatant was no longer green. The resulting BBY particles were then resuspended with 10% glycerin and used for further experiments. In BBY particles, 220-250 chlorophyll molecules account for one reaction center. The concentration of chlorophyll was determined in 96% ethanol (vol./vol.) according to the method described in (Arnon, 1949).

Photosynthetic oxygen evolution measurements: The rate of photosynthetic oxygen release was measured by the amperometric method under stationary conditions with continuous illumination at a Hansatech installation (Hansatech Instruments Ltd., Great Britain) using a Clark oxygen electrode combined with a temperature-controlled water bath according to the method (Renger & Hanssum, 2009). The chlorophyll concentration was 20 micrograms/ml. Measurements were carried out in a reaction buffer (0.4 M sucrose; 50 mM MES (pH 6.5); 10 mM NaCl; 5 mM $MgCl_2$; 10 mM $CaCl_2$). 1 mM 2,6-dichloro-p-benzoquinone (DCBQ) and 1mM potassium ferricyanide (FeCy) were used as artificial electron acceptors.

Photoinduced changes of the PSII chlorophyll fluorescence yield measurements: The photosynthetic activity of thylakoids and BBY particles was controlled by photoinduced changes in the fluorescence yield of chlorophyll (ΔF) associated with the photoreduction of the primary electron acceptor of PS II, plastoquinone QA. The values of variable fluorescence (F_V) were calculated using the equation: $F_V=F_M-F0$

where F_M is maximal fluorescence F0 is initial fluorescence. The measurements were carried out in a cuvette with a final volume of the reaction mixture of 2 ml. The concentration of chlorophyll was 20 micrograms/ml. The reaction mixture was incubated with copper (II) complexes for 3 minutes. DMSO (dimethyl sulfoxide) was added to the control samples in a concentration equal to the concentration of copper complexes.

Spectrophotometric measurements: The absorption spectra of the $[CuL_2]Br_2$ complex were recorded at room temperature using a Shimadzu UV-1800 dual-beam spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) in a standard quartz cell Hellma (Muhlheim, Germany) with an optical path length of 10 mm. The measurements were carried out in the wavelength range of 200-700 nm with a slit width of 2 nm and a scanning speed of 2 $nm \cdot s^{-1}$.

RESULTS

Effects of $[CuL_2]Br_2$ on the photosynthetic oxygen evolution: It is known that Cu(II) aqua ions are capable of (1) oxidizing a wide range of inorganic and organic compounds, (2) stimulating photosynthetic oxygen release at low concentrations and (3) possibly retaining these properties as part of the $[CuL_2]Br_2$ complex. In this regard, we investigated the ability of $[CuL_2]Br_2$ to act as an artificial electron acceptor in the photosynthetic oxygen release reaction in PSII. However, when $[CuL_2]Br_2$ was added in the concentration range from 1 to 30 mM (in the absence of other artificial electron acceptors, AEA), oxygen release by PSII membranes was not observed (Fig. 2, kinetics 6).Under control conditions, in the presence of DCBQ and FeCy acceptors, but without $[CuL_2]Br_2$ oxygen was released from the PSII membrane under the action of continuous saturating illumination at a rate of about 480 mmol $O_2 \cdot$(mg chlorophyll·h)$^{-1}$ (Fig. 2, kinetics 1).

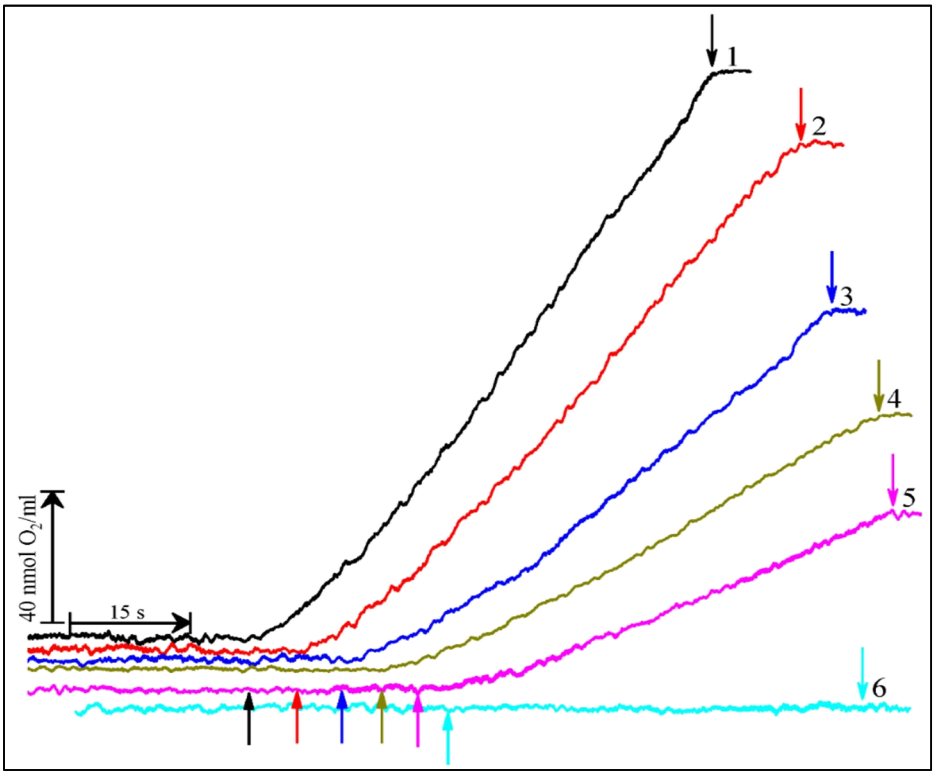

Fig. 2. Kinetics of the PSII membranes oxygen evolution in the absence of any

additions (1) and in the presence of $[CuL_2]Br_2$ at concentration of 3 μM (2); 20 μM (3); 50 μM (4); 100 μM (5). The assay medium contained 50 mM MES (pH 6.5), 0.33 M sucrose, 15 mM NaCl, 0.1 mM DCBQ, 1 mM FeCy. ↑ and ↓—light (λ = 650 nm, 1200 μmol photon s^{-1} m^{-2}) on and light off, respectively. The kinetic 6 was measured in the absence of artificial electron acceptors but in the presence of 30 μM $[CuL_2]Br_2$.

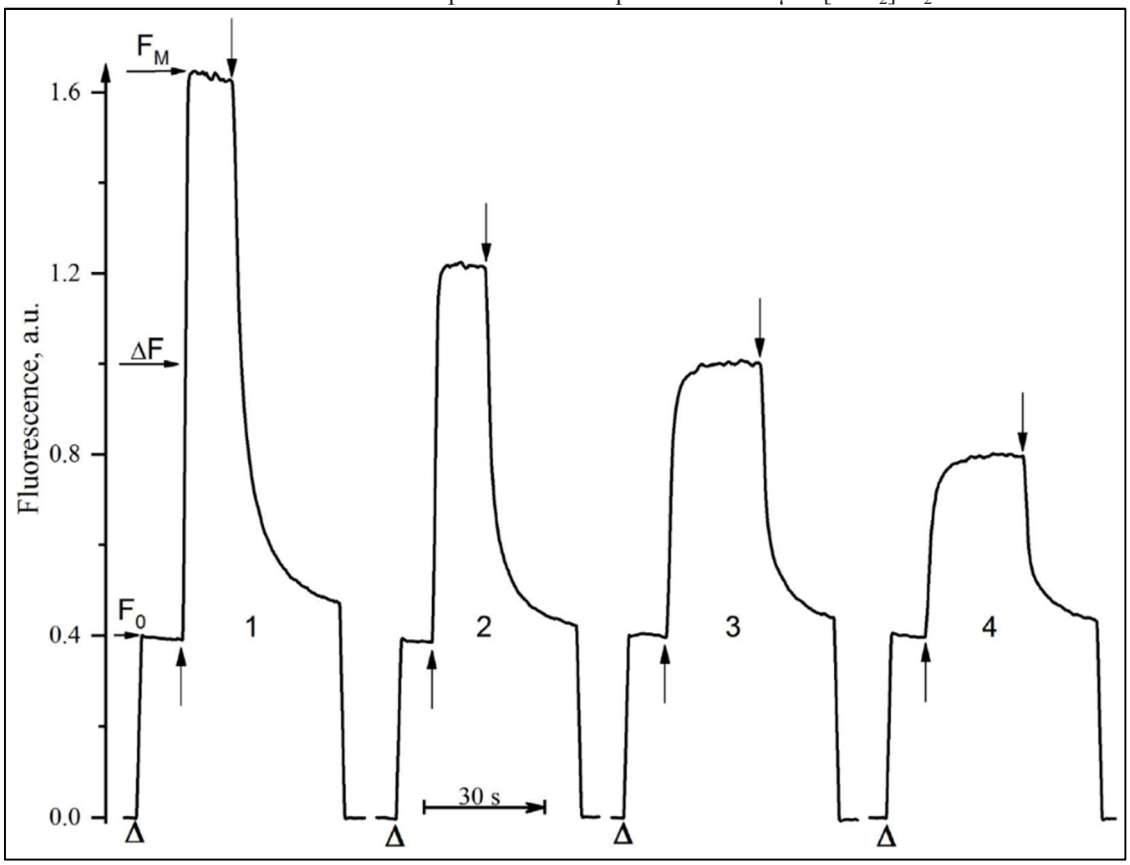

Fig. 3. Kinetics of the photoinduced changes of the PSII chlorophyll fluorescence yield (ΔF) related to the photoreduction of the primary quinone electron acceptor, Q_A, in PSII membranes in the absence of any additions (kinetics 1) and in the presence of $[CuL_2]Br_2$ at concentration of 8 μM (kinetics 2), 30 μM (kinetics 3) and 100 μM (kinetics 4). Triangle symbol indicates the moment of switching on the measuring light (λ=490 nm, 4 μmol photons m^{-2} s^{-1}) exciting PSII chlorophyll fluorescence, F_0 (λ≥650 nm). The upward ↑ and downward ↓ arrows indicate the moment of respective switching on and off the actinic light (λ>600 nm, 1000 μmol photons m^{-2} s^{-1}).

The addition of the $[CuL_2]Br_2$ complex resulted in a concentration-dependent inhibition of photosynthetic oxygen release. At concentrations of 3, 20, 50, and 100 mM, the reaction rate decreased on about 15%, 43%, 59%, and 69%, respectively, compared with the control (100%) (Fig. 2, kinetics 2-5). It should be noted that the degree of inhibition of photosynthetic activity of PSII membranes did not depend on the duration of their incubation (in the range of 5-20 min) in the presence of this complex.

Effects of $[CuL_2]Br_2$ on the photoinduced changes of the PSII chlorophyll fluorescence yield: The suppression of oxygen evolution during photosynthesis is considered one of the key signs that the compound under study actually inhibits the functional activity of photosystem II (PSII). Nevertheless, the results of our experiment do not allow us to unambiguously determine whether this agent (1) affects the donor or acceptor side of PSII or directly at the level of the reaction center (RC); (2) which intermediate component of the electron transport chain it affects.; and (3)

whether it manifests itself as an inhibitor of electron transport or as an external electron donor (or acceptor) capable of competing with the natural components of PSII and thereby disrupting the normal course of photochemical reactions. To clarify (a) the localization of the inhibition site in PSII, (b) the number and location of the main and additional centers of action, and (c) the possible mechanism of inhibition, an analysis of photoinduced changes in chlorophyll PSII (Fv) fluorescence yields can be used, which reflect the photoreduction processes of the primary quinone acceptor QA. This approach also includes the use of exogenous electron donors and acceptors, as well as known inhibitors with well-studied mechanisms of action on PSII. In this regard, this method was used in our work to study in more detail the localization and nature of the inhibitory effect of the $[CuL_2]Br_2$ complex on PSII.

The results of the experiments are shown in Figure 3. It shows the kinetics of photoinduced changes in chlorophyll PSII (Fv) fluorescence, reflecting the process of photoreduction of the primary quinone acceptor QA in particles of PSII subchloroplast membranes, both under control conditions (kinetics 1) and with the addition of the $[CuL_2]Br_2$ complex at concentrations of 8 mM (kinetics 2), 30 mM (kinetics 3) and 100 mM (kinetics 4).In these experiments, the parameters F_0 and F□ were defined as the minimum and maximum levels of chlorophyll *a* fluorescence in samples adapted to darkness. Accordingly, FV and the Fv/Fm ratio were used to evaluate possible changes in the photochemical activity of PSII under the action of the inhibitor compared with the control. In the control samples (without the addition of an effector), the Fv/Fm value was 0.79 ± 0.02, which indicates the high photochemical efficiency of PSII in the used sample.

The addition of the $[CuL_2]Br_2$ complex caused a noticeable decrease in the level of Fm (kinetics 2), and the level of decrease increased with increasing concentration of the compound (kinetics 3 and 4). It should be emphasized that at all the studied concentrations, the decrease in Fm occurred solely due to a decrease in FV, while the value of F0 remained unchanged (kinetics 2-4). Therefore, to quantify the inhibitory effect of $[CuL_2]Br_2$, FV was used as an indicator, expressed as a percentage relative to the control value.

The inhibition level of photoinduced FV was 66%, 48%, and 32% at concentrations of $[CuL_2]Br_2$ 8, 30, and 100 mM, respectively, which is consistent with the results of experiments measuring the rate of oxygen release (Fig. 2). In addition, several specific effects have been detected. At all the studied concentrations of the complex, (a) changes in the kinetics of the dark relaxation of Fm were observed, expressed in its acceleration or deceleration, reflecting the dark reoxidation of QA^-, and (b) the absence of a deceleration of the photoinduced increase of Fm.

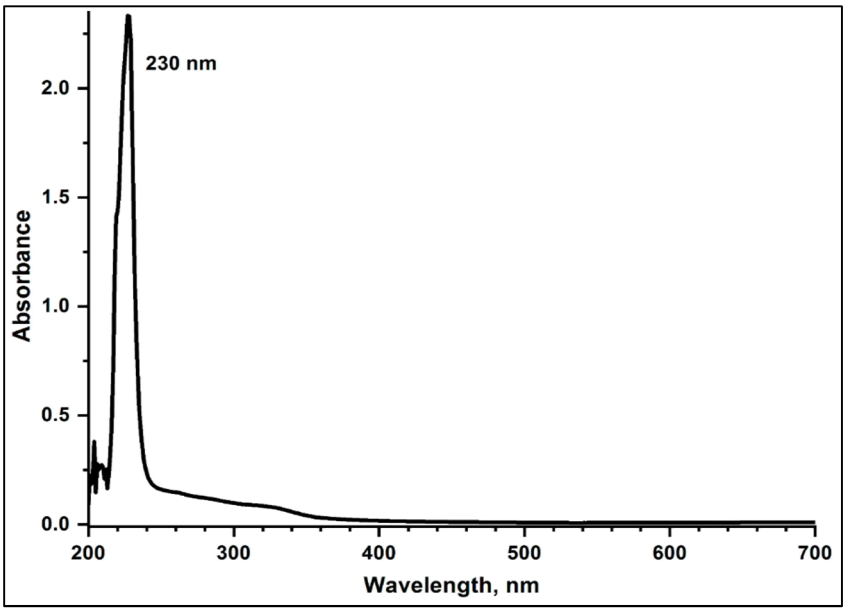

Fig. 4. Absorption spectrum of $[CuL_2]Br_2$ at a concentration of 0.1 mM in the range of 200–700 nm, in the medium for measuring OJIP-kinetics at room temperature.

Absorption spectrum of [CuL₂]Br₂: It can be assumed that the observed decrease in the amplitude of the kinetics of rapid induction of chlorophyll fluorescence and almost all of its characteristic peaks with the addition of the $[CuL_2]Br_2$ complex, the degree of which increases with increasing concentration of this compound, is due to its effect on the photochemical activity of PSII. Initially, this decrease in intensity could be explained by two possible reasons: (1) attenuation of the measuring and/or exciting light flux due to the shielding effect of the $[CuL_2]Br_2$ complex, that is, its ability to absorb photons; (2) partial absorption of the emitted fluorescence by the complex itself, that is, the effect of reabsorption of chlorophyll luminescence by $[CuL_2]Br_2$ molecules in the environment.

To verify this assumption, the absorption spectrum of the $[CuL_2]Br_2$ complex was recorded. As can be seen from the data presented in Figure 4, this complex does not exhibit noticeable absorption bands either in the wavelength range corresponding to the active and measuring light or in the emission region of chlorophyll fluorescence. Consequently, the above hypothesis about the effect of optical effects of the complex on the shape and intensity of fluorescence induction curves is not confirmed.

CONCLUSION

As a result, we can emphasize that the copper complex acts as an inhibitor on the photosynthetic activity of plants. The copper complex can suppress the photosynthetic activity of photochemical processes such as oxygen evolution and the PSII photoinduction chlorophyll fluorescence yield. The data obtained can be useful for further development of new types of herbicides.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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