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Ultraviolet Rays Promote Development of Photosystem II Photochemical Activity and Accumulation of Phenolic Compounds in the Tea Callus Culture (*Camellia sinensis*)

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Abstract—Effect of UV-B rays (280–320 nm) on photosynthetic electron transport and production of phenolic compounds in tea (*Camellia sinensis* L.) callus culture grown in white light was investigated. When white light was supplemented with UV radiation, the culture growth was retarded and morphological characteristics were modified. These conditions promoted the formation of chlorophyll-bearing cells and altered the ability of cultured cells to accumulate phenolic compounds, including flavans specific to *Camellia sinensis*. By the end of the culturing cycle (on the 45th day), the total content of phenolic compounds in the culture grown under supplementary UV irradiation was almost 1.5 times higher than in the control culture. The UV rays greatly stimulated photosystem II (PSII) activity in phototrophic cells of the callus culture, which was indicated by a large increase in the ratio of variable chlorophyll fluorescence to maximal fluorescence. This ratio was as low as 0.19 in cells cultured in white light and increased to 0.53 in the cell culture grown under white and UV light. The kinetics of dark relaxation of chlorophyll variable fluorescence, related to reoxidation of PSII primary acceptor, contained either two or three components, depending on the absence or presence of UV radiation, respectively. An artificial electron acceptor of PSI, methyl viologen modified the kinetics of dark decay of chlorophyll variable fluorescence in a characteristic manner, implying that photosynthetic electron transport was mediated by PSI and PSII in both treatments (culturing in white light with and without UV-B). It is concluded that stimulatory effect of UV rays on the parameters examined in phototrophic regions of *Camellia* tissue culture is determined by photoexcitation of a regulatory pigment that absorbs quanta in blue and long-wave UV spectral regions.

Key words: Camellia sinensis - tissue culture - phenolic compounds - chlorophyll fluorescence - photosystem II - ultraviolet light

INTRODUCTION

Light is among the most important factors of plant life, as it is needed for photosynthesis. The efficiency of this process affects the content of metabolites produced in primary and secondary metabolic pathways. Phenolic compounds, the products of secondary metabolism are indispensable components of all plant cells. The functions of phenolic compounds in plants are extremely diverse. For example, ubiquinones and plastoquinones are universal components of mitochondrial and photosynthetic electron transport chains [1]. Lignin, a complex polymer of phenolic origin provides mechanical rigidity to plant cells and tissues and ensures their viability under some stressful treatments [2]. Phenylpropanoids and flavonoids protect the photosynthetic and genetic machineries against the short-wave UV-B radiation [3, 4]. This spectral range of solar radiation (280-320 nm) is among the strongest stress factors for plants [1, 5]. It is established that short-wave UV light suppresses plant growth [6, 7], modifies the hormonal status [8], perturbs the development of leaf apparatus, including the chloroplast structure [9], and affects synthesis of phenolic compounds [10]. While phenolic compounds are important for plant resistance to UV light, the mechanisms by which UV rays affect photosystem functioning and consequent accumulation of phenolic compounds in plant cells are far from clear.

A convenient model for exploring these issues is *in vitro* cultures that are grown under strictly controlled conditions and retain the ability of producing phenolic compounds characteristic of the intact plant [11, 12]. Such cultures proved useful for elucidating many aspects of phenolics biosynthesis and regulation, including the transition to autotrophic nutrition upon the development of chloroplasts within the cells [13]. However, there is no conclusive evidence to date whether or not chlorophyll molecules in callus and suspension cultures constitute the functionally active pho-

Abbreviations: PSI and PSII—photosystems I and II.

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tosynthetic apparatus capable of supporting linear electron transport. It is well known that chlorophyll molecules are heterogeneous with respect to their functions: some of them are components of the light-harvesting complexes and do not possess photochemical activity, while other chlorophylls are the key components of photosystem (PS) reaction centers and directly initiate photosynthetic electron flow [14]. In the case of *in vitro* cultures with chlorophyll-containing cells lacking PSII and PSI reaction centers, the chlorophyll accumulation is apparently unrelated to any significant function.

One of the most reliable methods for detecting the functionally active reaction centers of PSII is based on recordings of light-induced changes of chlorophyll variable fluorescence [15]. Furthermore, the ratio of variable fluorescence to maximal fluorescence is a conventional measure of PSII activity [15]. When the plant samples containing PSII are exposed to light, the quantum yield of chlorophyll fluorescence increases severalfold owing to photoreduction of primary acceptor of PSII [16]. In samples devoid of PSII reaction centers, there is no variable fluorescence despite the presence of chlorophyll in cells [17]. Thus, the analysis of light-induced changes in chlorophyll fluorescence allows not only qualitative detection of PSII reaction centers in plant samples but also enables quantitative characterization of this photosystem.

In growing plant objects, light performs dual function by ensuring both photosynthesis and photoregulatory events sensitized by specific pigments [18]. One of the best-known photoreceptor of phototrophic cells is a pigment absorbing light in the blue and long-wave UV spectral range [19]. Numerous studies have shown that regulatory responses sensitized by this pigment exert stimulating action on the development of photosynthetic apparatus [20–22].

The application of blue light in experiments with photosynthesizing cells and organisms is associated with certain methodic difficulties. Apart from excitation of specific photoreceptor, blue light is absorbed by chlorophyll molecules and drives photosynthesis. In order to separate the substrate and regulatory functions of blue light, it is necessary to investigate the action of blue light in a wide range of intensities [23]. Unlike blue light, UV radiation is photosynthetically inactive and can be safely applied to induce photoregulatory responses.

This study aimed at exploring the effects of UV-B radiation on photosynthetic electron transport in autotrophic regions of photomixotrophic callus cultures of *Camellia sinensis.* In addition, we used calluses to study the effects of UV-B light on accumulation of phenolic compounds involved in protection of cells against harmful action of UV radiation.

MATERIALS AND METHODS

Experiments were performed with the callus culture (strain ChS-2) initiated from the stem of tea plant (*Camellia sinensis* L., Georgian variety). The culture was grown on a modified Heller nutrient solution supplemented with 2,4-dichlorophenoxyacetic acid (5 μg/l) and glucose (25 g/l) [24]. Calluses were grown at 26°C and 16-h photoperiod (white light, 4 klx) in the absence (control) and presence (treatment) of supplementary UV-B radiation (280–320 nm). The UV radiation was provided from DRLF-400 lamp, the outer bulb of which was removed. This light source is equivalent to bactericidal high-pressure mercury lamp PRK-2 (DRT). The intensity of UV-B radiation was 0.74 W/m^2. The culture was irradiated daily for 2 h in the period from 4:00 to 6:00 p.m. throughout the culturing cycle (7 weeks).

The callus growth was evaluated from changes in callus fresh weight on the 25th, 35th, and 45th days of culturing; i.e., in the middle and in the end of linear growth stage and at the stationary growth stage, respectively.

The treatment with methyl viologen was accomplished by immersing callus samples into 1 mM solution for 30 min in darkness.

Chlorophyll fluorescence was measured with a specialized PAM-101 fluorometer (Walz, Germany) connected to a computer via a PDA-100 Data Acquisition System. In the beginning of each experiment, the sample predarkened for 30 min was illuminated with weak light in order to measure so-called background fluorescence. Next, the sample was illuminated with a light pulse of either 2 s or 50 ms duration in order to induce variable fluorescence [25]. A 2-s pulse of strong white light was obtained from a KL-1500 light source (Schott, Germany) equipped with a shutter. A short pulse with a length of 50 ms was obtained from a xenon lamp connected to a dedicated power supply (Walz).

Phenolic compounds were extracted from fresh samples with hot 96% ethanol. The total content of soluble phenolic compounds in ethanol extracts was measured with the Folin–Denis reagent (absorbance at 725 nm), and the flavan content was determined with a vanillin reagent (absorbance at 500 nm) [26]. In both cases, the standard calibration curves were plotted using (–)-epicatechin.

Data in figures represent mean values and standard deviations calculated from three replicates (each replicate contained 3–5 calluses) analyzed throughout two cycles of subculturing.

RESULTS

The callus culture from a stem of *Camellia sinensis* grown under 16-h illumination with white light appeared as a dense compact yellow callus whose surface contained green zones gradually expanding by the

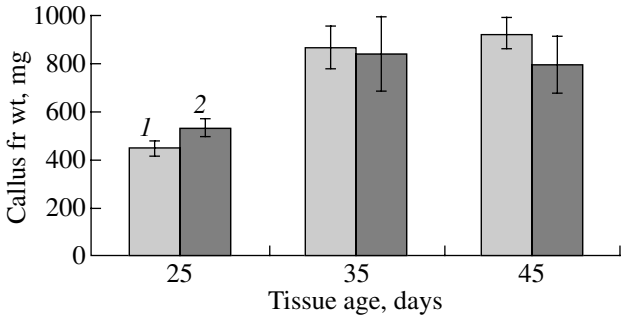

Fig. 1. Changes in fresh weight of *Camellia sinensis* calluses grown in (*1*) white light and (*2*) white light with supplemental UV radiation.

end of the passage. The green color of the cells was determined by the presence of chlorophyll and of developing chloroplasts in the calluses cultured under light [27].

Calluses grown under white light with supplementary UV rays were also compact and dense but yellow-green. The formation of chlorophyll-containing cells proceeded faster in these calluses than in the control culture; the difference was especially evident in the first half of the growth cycle (until the 25th day).

The growth rates of tea callus cultures are characteristically very low [28]. As shown in Fig. 1, the callus weight for both treatments increased significantly in the period from the 25th to 35th days of culturing. During further culturing the biomass increased only in the control callus. Hence, UV radiation facilitated earlier completion of the growth cycle and promoted the transition of the culture to the stationary growth.

The tea callus cultures retain the ability of whole plants to synthesize phenolic compounds, including flavans that exhibit P-vitamin and capillary stabilizing activities [24, 29]. In the culture grown in UV-free white light, the largest accumulation of flavans was noted on the 25th day (Fig. 2). By the 35th day, the total contents of phenolic compounds and flavans reduced by 38 and 35%, respectively. Further culturing (until the 45th day) had no significant influence on the level of phenolic compounds.

In the culture irradiated with UV during growth, the accumulation pattern of phenolic compounds was clearly different. The largest total content of soluble phenolic compounds was noted by the end of the growth cycle (45th day). The amount of flavans was high in the 25-day-old culture; by the 35th day it decreased almost twofold, and by the 45th day the flavan level increased again. All these observations demonstrate the differences in the production of phenolic compounds in the tea callus cultures grown with and without UV irradiation. Based on the notion that synthesis of phenolic compounds is related to functioning of chloroplasts [30], we investigated photosynthetic activity of the cultured cells.

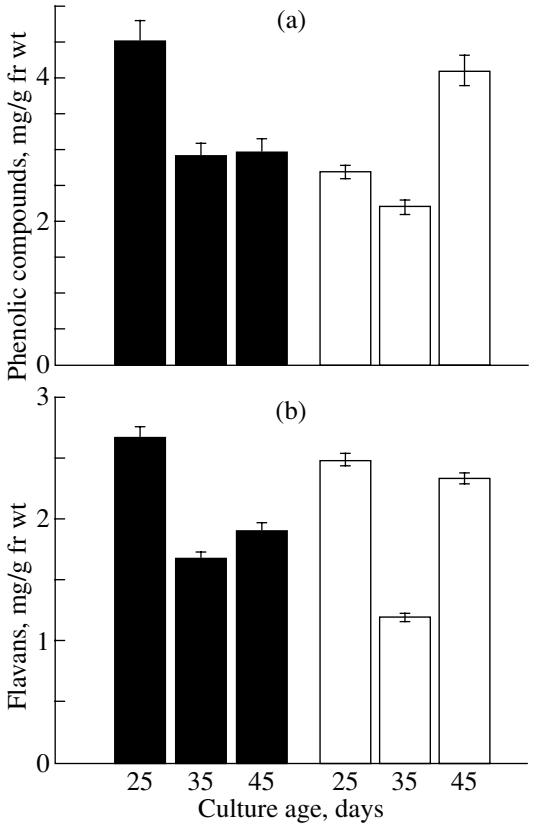

Fig. 2. Total content of (a) soluble phenolic compounds and (b) flavans in the tea callus culture grown in white light without (■) or with (□) supplemental UV radiation.

Figure 3 shows kinetic curves of chlorophyll fluorescence changes induced by 2-s pulse of white light in cultured tissues unexposed or exposed to UV rays during callus growth. Because of complicated callus geometry, we could not prepare samples of standard area; therefore, we normalized signals to the amplitude of the background fluorescence detected under weak measuring light. This scaling procedure was justified because the absolute amplitudes of the measured fluorescence signal provide no direct information on properties of photosynthetic apparatus. On the other hand, the ratio of variable fluorescence to maximal chlorophyll fluorescence is a well-known characteristic of PSII activity for dark-adapted samples. This parameter characterizes the potential quantum yield of primary charge separation in PSII [18].

In calluses grown without UV irradiation, the increase in chlorophyll fluorescence under illumination with high-intensity white light produced only a small increase in chlorophyll fluorescence (Fig. 3a, curve *1*). The ratio of variable fluorescence to maximal fluorescence was 0.19. By contrast, in samples exposed to UV rays during growth, the amplitude of variable fluorescence was much larger, and the ratio of variable to max-

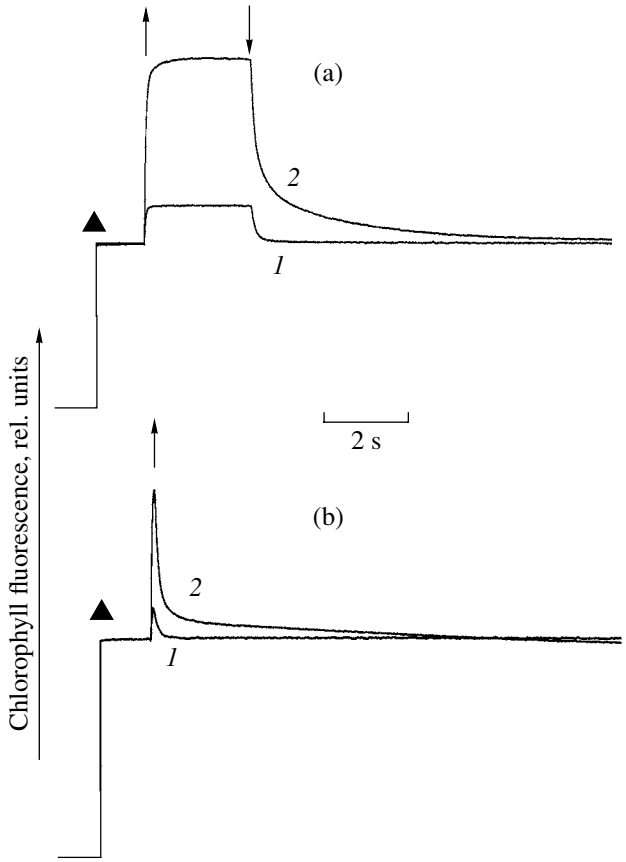

Fig. 3. Kinetic curves of chlorophyll fluorescence induced by (a) 2-s pulse of white light and (b) 50-ms flash of xenon lamp in the tea callus culture grown (*1*) in white light and (*2*) white light with supplemental UV radiation.

Triangles (▲) designate the onset of weak measuring light. Upward arrows mark the moments when the actinic light was turned on and when the 50-ms flash was fired; downward arrows show the moment of switching off the actinic light.

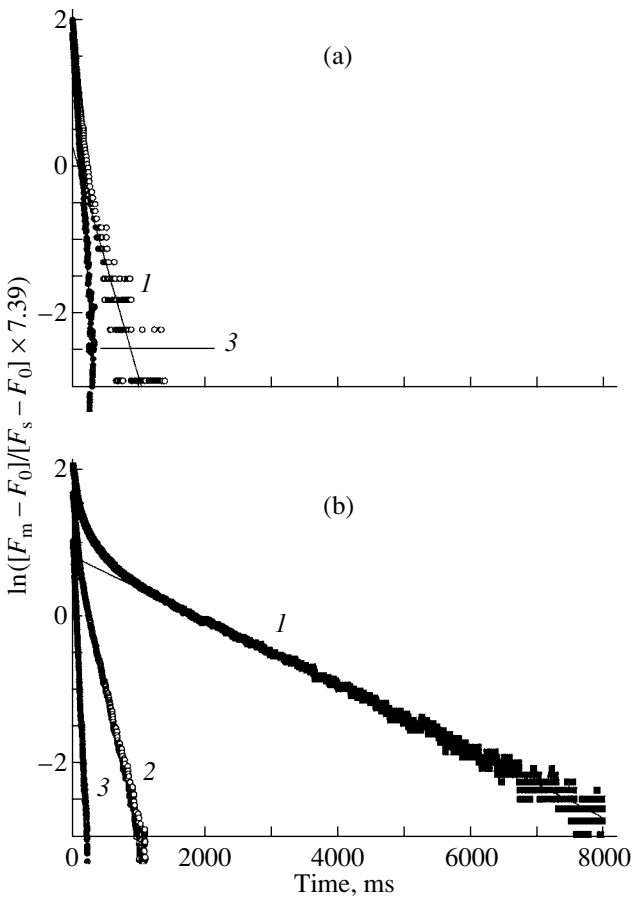

Fig. 4. Semi-logarithmic plots of kinetic curves of dark relaxation of chlorophyll variable fluorescence induced by 2-s pulse of actinic light in tea callus culture grown in light (a) without and (b) with supplemental UV radiation.

(*1*) Initial kinetic curves, (*2*) middle component, and (*3*) fast component. F_m, F_0, and F_s designate respectively the maximal, background, and stationary fluorescence levels.

imal fluorescence levels equaled to 0.53, which indicates a higher PSII activity (Fig. 3a, curve *2*).

Apart from different amplitudes of variable fluorescence in untreated and UV-irradiated cultures, the kinetic curves of dark decay of variable fluorescence were clearly different, reflecting dissimilar patterns of reoxidation of PSII primary acceptor [31]. These distinctions were most evident when the kinetic curves were decomposed into individual components [32, 33], as shown in Fig. 4. This figure shows the decay of variable fluorescence plotted in semi-logarithmic coordinates, when the variable fluorescence of each sample was normalized to its maximal amplitude. The decay of variable fluorescence in the control culture consisted of two exponential terms. The relative extent and half-decay time of slowly decaying component were 14% and 224 ms, respectively (Fig. 4a, curve *1*). The extent and half-decay times for the fast kinetic component were 88% and 49 ms, respectively (Fig. 4a, curve *2*). In the callus culture grown under supplemental UV-B, the dark decay of variable fluorescence was more complex than in the control culture. The decay kinetics of these samples comprised three exponential terms having similar magnitudes and strikingly different relaxation times. The slow component had the relative extent of 30% and a half-decay time of 1580 ms (Fig. 4b, curve *1*). The middle (Fig. 4b, curve *2*) and fast (Fig. 4b, curve *3*) kinetic components measured 32% and 36% of the total amplitude, and their decay halftimes were 208 and 43 ms, respectively.

Earlier studies showed that the multicomponent kinetics of dark relaxation of chlorophyll variable fluorescence reflects the existence of several populations of PSII reaction centers that are connected to plastoquinone pools with different extents of reduction [32, 33]. In order to diminish the extent of plastoquinone pool reduction, we examined the signals of variable fluorescence induced by relatively short

(50 ms) flash of white light (Fig. 3b). The amplitude of variable fluorescence induced by 50-ms flash was much higher in tissues grown under supplemental UV radiation than in tissues of control calluses, which is similar to observations with longer light pulses. However, the kinetic curves of the dark decay of variable fluorescence measured after 2-s and 50-ms light exposures were strikingly different for both treatments.

In the tissue culture grown without UV irradiation, the kinetics of dark decay of variable fluorescence after a 50-ms flash was monoexponential with a decay halftime of 58 ms (Fig. 5a). In the culture grown under supplemental UV irradiation, three exponential components were distinguished in the dark decay after the same flash illumination, in accord with the situation observed after longer light pulses (Fig. 5b). The amplitude of the slow component with a decay halftime of 6100 ms was reduced in this condition and constituted only 16% (Fig. 5b, curve *1*). In the dark decay of variable fluorescence after the 50-ms flash, the amplitude of the middle component with a decay halftime of 168 ms amounted 29% (Fig. 5b, curve *2*), which was virtually identical to its contribution observed after 2-s illumination. By contrast, the amplitude of the fast component (decay halftime 34 ms) increased up to 58% of the total signal of variable fluorescence (Fig. 5b, curve *3*).

It is well known that the rate of linear electron transport is often limited by the outflow of electrons from PSI. This situation arises due to low activity of the terminal component in electron transport chain, ferredoxin–$NADP^+$ oxidoreductase [34] or due to slow consumption of NADPH in the Calvin cycle [35, 36]. In order to assess possible influence of this factor on electron transport in the case of tea callus culture, we examined light-induced changes of chlorophyll fluorescence in tissue culture samples treated with methyl viologen, an artificial electron acceptor of PSI. Methyl viologen accepts electrons with high affinity from bound iron–sulfur centers at the acceptor side of PSI, thereby bypassing the possible bottleneck limitation at this segment of electron transport chain [37]. Figure 6a shows that the treatment of tissues with methyl viologen did not eliminate distinctions in the amplitude of variable fluorescence for callus cultures grown with and without UV irradiation. At the same time, such treatment had a strong influence on the kinetics of dark decay of variable fluorescence.

After 2-s actinic illumination of tissue culture samples treated with methyl viologen, the kinetics of dark decay of variable fluorescence was monoexponential, irrespective of UV irradiation of calluses during their growth (Fig. 6b). The decay halftimes were 78 and 69 ms for the samples nonirradiated (Fig. 6b, curve *1*) and irradiated (Fig. 6b, curve *2*) with UV light, respectively.

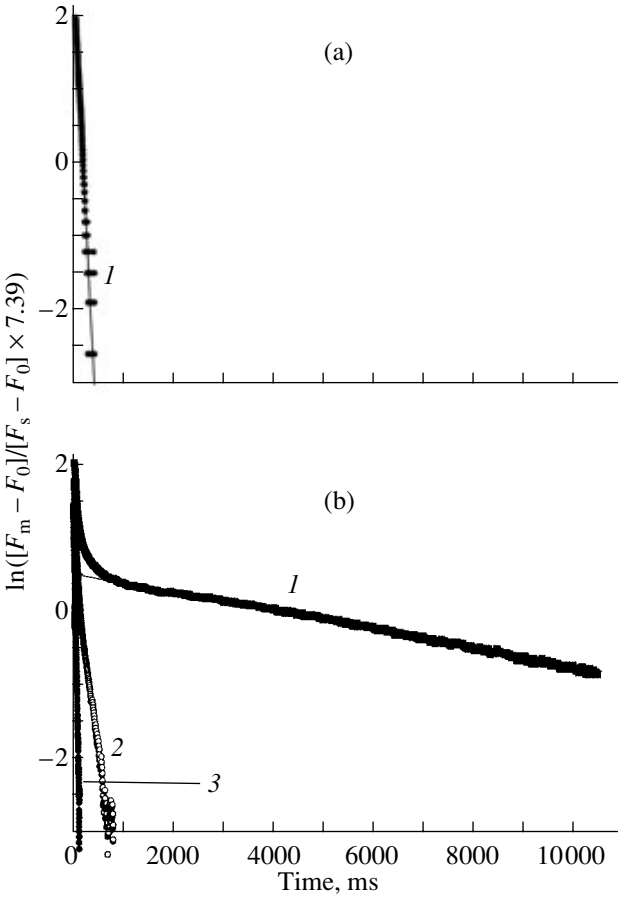

Fig. 5. Semi-logarithmic plots of kinetic curves for dark relaxation of chlorophyll variable fluorescence induced by 50-ms flash in the callus culture grown in light (a) without and (b) with supplemental UV radiation.
(*1*) Initial kinetic curves, (*2*) and (*3*) deconvoluted middle and fast kinetic components. See Fig. 4 for designations.

DISCUSSION

Plant callus cultures proved useful for studying the mechanisms of cell protection against stress factors. In particular, calluses of *Rosa damascena* provided the first convincing evidence that the resistance of cells to UV radiation is related to their capacity of accumulating phenolic compounds [38]. In the case of callus cultures of tea plants, a strain highly productive for phenolic compounds was more resistant to UV radiation than the low-productive strain [39].

It is known that changes in light quality or intensity usually affects morphophysiological and biochemical characteristics of plant cells [14, 19, 22, 40]. The UV irradiation supplemental to white light also altered these characteristics. The effect of UV light was manifested in formation of structurally more compact calluses and in deceleration of callus growth (Fig. 1); these alterations could be related to diminished cell sizes, as previously reported [39]. The retardation of growth

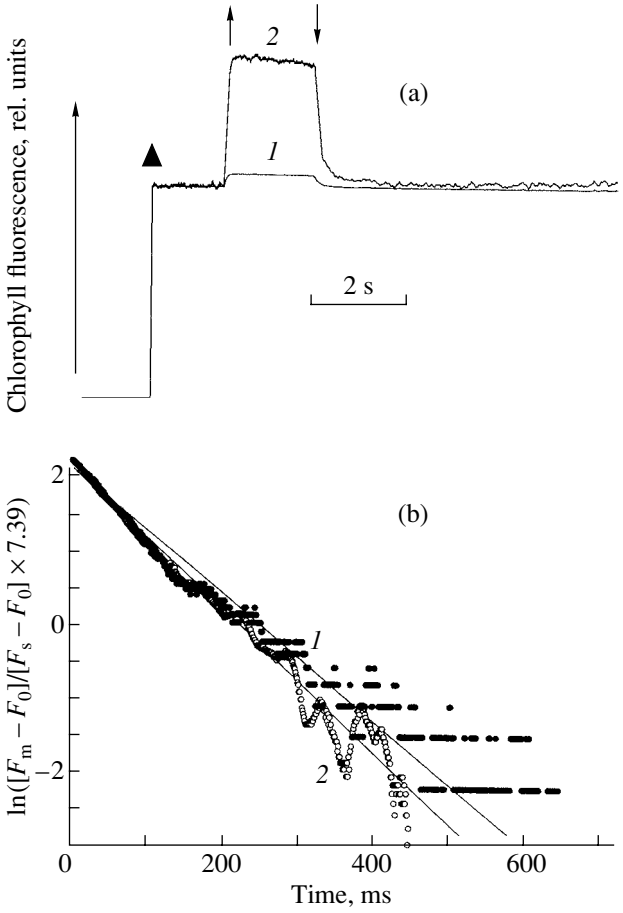

Fig. 6. Kinetic curves of (a) chlorophyll fluorescence changes induced by 2-s pulse of white light and (b) semilogarithmic plots for the kinetics of dark relaxation of variable fluorescence in the callus cultures grown in (*1*) white light and (*2*) white light with supplemental UV radiation. Other designations are the same as in Figs. 3 and 4.

under the action of UV radiation is characteristic of most plant objects [20].

The UV irradiation modified the ability of calluses to accumulate phenolic compounds (Fig. 2). Under control conditions, the total content of soluble phenolic compounds and flavans peaked on the 25th day of culturing and then decreased. In calluses treated with UV, an opposite trend was noted; for example, the total content of phenolic compounds turned out maximal by the end of the passage. This indicated, in consistency with repeated statements in the literature [12], that UV rays promote accumulation of phenolic compounds in cells that completed their growth.

The exposure of callus culture during growth to supplemental UV radiation affected the fraction of flavans (the major components of the phenol complex in tea plants) in the total balance of phenolic compounds. For example, the relative content of flavans in the total amount of phenolic compounds was 50–60% under control conditions, while it reached 80% in calluses exposed to UV rays. All these findings indicate that biosynthesis of phenolic compounds (phenylpropanoids and flavonoids) in the tea calluses was modified not only as a result of culture growth but also due to UV irradiation; this view is compatible with published data [41]. It is presently established that numerous components of phenol metabolism, including phenylpropanoids and flavonoids can protect cell against damaging action of UV radiation, including the short-wave UV-B region that is often perishing for plants [5].

We showed earlier that the phenol complex in photomixotrophic (chloroplast-bearing) tea callus cultures contains mainly phenylpropanoids and flavonoids represented by flavans and flavonols [29]. The synthesis of flavans persisted under various culturing conditions, whereas the flavonol synthesis occurred only under the action of white light. In fact, the flavonol synthesis began only after chloroplast formation, since a large part of phenol production in green plants is confined to chloroplasts [1, 29, 30]. It is possible that development of green chlorophyll-containing cells in the callus cultures of tea plants, which proceeded faster under the action of UV rays, facilitated the activation of synthesis of these phenolic compounds.

The callus cultures grown in light with and without supplemental UV radiation demonstrated light-induced signals of variable chlorophyll fluorescence (Fig. 3). This is a clear evidence for the existence of photochemical activity of PSII, as noted also by other authors [42, 43]. The amplitude of variable fluorescence was much higher in UV-irradiated calluses than in nontreated cultures. Thus, UV rays not only stimulated the formation of chlorophyll-containing cells but also promoted development of photosynthetic apparatus in autotrophic parts of the callus culture. Although we did not measure electron transport in PSI by direct methods, the characteristic effect of methyl viologen on the kinetics of dark decay of variable fluorescence (Figs. 3, 4), analogous to its effect in higher plant leaves [32, 33], clearly indicates the occurrence of electron flow between PSII and PSI. In this context, there was no qualitative difference between the tissue cultures grown under dissimilar conditions. At the same time, different kinetics of the dark decay of variable fluorescence clearly show the specific influence of UV light on electron efflux from the reduced primary acceptor of PSII.

The slow component in the dark decay of variable fluorescence is known to reflect reoxidation of primary acceptor in PSII reaction centers associated with fully reduced pool of plastoquinones [32, 33]. This component in the dark decay of variable fluorescence was only observed in the culture grown under UV irradiation, and the amplitude of this component increased significantly after a longer light pulse (Figs. 3, 4). Methyl viologen removed completely both the slow and the middle components in the kinetic curves of the dark decay of variable fluorescence. Thus, the origin of the slow component and the relatively small amplitude of

middle component in autotrophic cells of the culture grown in the absence of UV rays points to a lower probability for the plastoquinone pool to be photoreduced in this culture. The lower extent of plastoquinone reduction could be caused by faster electron drainage from the plastoquinone pool to PSI and by slower electron input to this pool from PSII. The fast component in the dark decay reflects the electron efflux from the primary acceptor in the population of PSII reaction centers associated with fully oxidized pool of plastoquinones [32, 33]. The rates of this component were equal for both treatments. Therefore, we conclude that the electron flow from the reduced pool of plastoquinones was retarded in the UV-treated culture.

The regulatory effect of UV radiation has been considered above. In addition to regulatory influence of UV light, this radiation exerts also inhibitory action on electron flow in chloroplasts, especially in PSII [43]. This inhibition is caused by the destruction of either PSII reaction center or water-splitting complex. In our case, the inhibitory action of UV was apparently absent, because the chlorophyll accumulation and the development of PSII photochemical activity were accelerated in cultures subjected to UV radiation. Meanwhile, the stimulatory effect of UV radiation was manifested not only in the development of photosynthetic machinery but also in accumulation of phenolic compounds. One of the documented functions of plant phenols is the protection of tissues against deleterious consequences of UV irradiation through the absorption of UV rays in the surface cell layer [44]. Thus, we can conclude that the protective mechanisms are mobilized already at rather low levels of UV irradiation that are not damaging to cells but are supposedly sufficient to cause photoregulatory responses. Apparently, this phenomenon should be taken into account in the context of adaptation of plant cells to elevated levels of UV radiation.

Our results revealed the occurrence of noncyclic electron transport in phototrophic regions of tissue cultures. This finding suggests the possibility that photosynthesis contributes to general metabolism of cultured cells and participates in biosynthesis of phenolic compounds. It is known that photosynthesis is associated with the production of precursors and intermediates of phenolic compounds. These include erythrose-4-phosphate, phosphoenolpyruvate, and acetyl-CoA. In addition, photosynthetic electron transport is associated with the production of energy (ATP) and reduced equivalents (NADPH) that are urgently needed for synthesis of phenolic compounds. It is remarkable that UV light promoted photosynthetic electron transport and expansion of green area occupied by chlorophyll-containing cells on the callus surface. Hence, the activated photosynthesis can be undoubtedly among the factors facilitating higher accumulation of phenolic compounds in the culture grown under supplemental UV irradiation compared to cultures grown under standard light conditions.

Finally, we conclude that the growing of tea plant tissue cultures in the light is accompanied by the formation of phototrophic cells on the callus surface. These phototrophic cells can support noncyclic electron transport, which may certainly contribute to tissue metabolism. A sharp rise in photochemical activity of phototrophic cells and profound accumulation of chlorophyll and phenolic compounds in these cells under combined application of white light and UV-B radiation is apparently due to photoregulatory effect of UV rays. The increased content of phenolic compounds in cultured cells of tea plant can be considered as the initial stage of plant acclimation to increased levels of UV radiation.

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