

# Epigallocatechin Gallate: pH-Dependent Redox Properties and Effect on Respiration, Photosynthesis, and Cell Death in Pea Plants

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**Abstract**—*In vitro* redox properties of the green tea component epigallocatechin gallate (EGCG) and its effect on pea plant cells were investigated. EGCG was found to exhibit both pro- and antioxidant properties. In solutions, EGCG was oxidized by oxygen at physiological (slightly alkaline) pH values with the generation of  $O_2^{\cdot-}$  and  $H_2O_2$ , the reaction being slowed down by a decrease in the medium pH. On the other hand, EGCG functioned as an electron donor for peroxidase, resulting in the  $H_2O_2$  utilization. EGCG suppressed respiration, reduced mitochondrial transmembrane potential difference and inhibited electron transfer in the photosynthetic electron transport chain in pea leaf cells (leaf cuttings and epidermis). Among components of the photosynthetic redox chain, Photosystem II was the least sensitive to the EGCG action. In the epidermis, EGCG reduced the rate of reactive oxygen species formation that was induced by NADH. EGCG at the concentrations from 10  $\mu$ M to 1 mM suppressed the KCN-induced death of guard cells in the epidermis, which was determined from the destruction of cell nuclei. EGCG at a concentration of 10 mM disrupted the barrier function of the guard cell plasma membrane, increasing its permeability to propidium iodide.

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**Keywords:** EGCG, peroxidase, reactive oxygen species, cell death, pea

## INTRODUCTION

Epigallocatechin gallate (EGCG), also known as epigallocatechin-3-gallate, is a compound from the catechin group of flavonoids (plant polyphenols) (for the structure and properties of catechins and EGCG, see the review by M. Farhan [1]). Dried tea leaves are the main source of catechins and EGCG. The highest content of EGCG can be found in green tea leaves; the concentration of EGCG in brewed green tea is 20-30 mg per 100 ml (approx. 0.5 mM).

It has been shown that green tea can prevent cancer development. The study conducted in Japan [2] demon-

strated that the risk of cancer is decreased in people, especially women, drinking more than 10 cups of green tea daily [2]. This effect of green tea is believed to be due to EGCG, whose anticancer activity has been observed in different types of tumors [3, 4].

EGCG exhibits various effects at the molecular and cellular levels. It exhibits antioxidant properties *per se* or as a result of activation of antioxidant enzymes in the cells [3, 5, 6]. Many proteins have to found to be EGCG-sensitive proteins, including enzymes related to tumor development and regulating: (i) the cell cycle (cyclins, cyclin-dependent and other regulatory protein kinases, transcription factors), (ii) apoptosis (Bcl-2 proteins,

**Abbreviations:**  $\Delta\psi$ , transmembrane potential difference; Amplex Red, N-acetyl-3,7-dihydroxyphenoxazine; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; EGCG, epigallocatechin gallate; ETC, electron transport chain; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species; TMPD, N,N,N',N'-tetramethyl-*p*-phenylene-diamine; TMRE, tetramethylrhodamine methyl ester.

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caspases, Apaf-1, XIAP), and (iii) inflammation (cytokines, NO synthase). Some of them directly interact with EGCG [3, 7]. For example, EGCG binds to the N-terminal region of the p53 tumor suppressor, preventing its contact with the E3 ubiquitin ligase MDM2, followed by p53 ubiquitination and degradation [8].

EGCG has been demonstrated to produce a therapeutic effect in a number of infectious viral diseases. The mechanisms of the EGCG antiviral activity can vary depending on the infectious agent, e.g., it can target the viral envelope. EGCG exhibits the antibacterial effect by itself or in combination with different antibiotics [9]; it is also effective against pathogenic fungi. In yeasts and Gram-negative bacteria, EGCG disrupts the metabolism of folic acid, thus mimicking the effects of sulfonamides and trimethoprim [9]. EGCG displays the antiviral effect against SARS-CoV-2 (COVID-19 pathogen). According to molecular modeling, EGCG interacts with the receptor-binding domain (RBD) of the viral S protein and disrupts its binding to the angiotensin-converting enzyme 2 (ACE2) on the cell surface [10].

In plants, flavonoids, including EGCG, are known regulators of ontogeny, reproduction, cell death, and interaction with symbiotic microorganisms [11]. They provide plant defense against pathogens and protect plant cells from UV radiation [12]. However, little is known about the proteins involved in the interaction with flavonoids in the cells, as well as on the effects of EGCG on plant cells. It has been reported that EGCG increases plant resistance to infections by activating jasmonic acid signaling pathway, which protects against necrotrophic pathogens [13].

In neutral and alkaline solutions, EGCG is oxidized with the generation of reactive oxygen species (ROS) [14]. In other words, EGCG demonstrates both antioxidant and prooxidant properties.

The goal of this work was to investigate the redox properties of EGCG *in vitro*, to elucidate the influence of the incubation medium and pH on these properties, and to test the effects of EGCG on the pea plant leaf cells, in particular, on the functions related to energy metabolism [respiration, generation of transmembrane potential difference ( $\Delta\psi$ ) in the mitochondria, and photosynthesis], ROS generation, and programmed cell death (PCD). Will the pro- or antioxidant properties of EGCG dominate in its effect on plant cells?

## MATERIALS AND METHODS

**Oximetry, respiration, and photosynthesis.** The  $O_2$  level in solution was recorded in a 1.5-ml polarographic cell with a closed platinum electrode connected to a Record4 analog-to-digital converter (Pushchino, Russia) using provided software. The  $O_2$  consumption during respiration and the photosynthetic release of  $O_2$  were deter-

mined. The composition of the incubation medium and added reagents are described in the legends to the figures.

**$H_2O_2$  production.** The  $H_2O_2$  content in solution was measured by fluorimetry using N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). The reaction between non-fluorescent Amplex Red and  $H_2O_2$  is catalyzed by peroxidase and leads to the formation of fluorescent resorufin [15, 16]. The reaction was conducted in a plastic cuvette with 2 ml of the incubation medium. Resorufin fluorescence was detected with a VersaFluor fluorimeter (Bio-Rad, USA) at  $\lambda_{ex} = 505\text{--}515$  nm and  $\lambda_{em} = 585\text{--}595$  nm.

**Plants.** Pea seedlings (*Pisum sativum* L. cv. Alpha) were grown for 9-17 days under periodic illumination (16 h/8 h light/dark cycle) with a DRiZ metal halide lamp (250 W) at a light intensity of  $\sim 100 \mu E m^{-2} \cdot s^{-1}$  (measured with a Quantitherm PAR/Temp sensor; Hansatech, Great Britain). Leaf cuttings were cut out of pea leaves with a plastic tube. The epidermis was separated from the lower leaf surface with forceps.

**Mitochondrial transmembrane potential  $\Delta\psi$**  was evaluated from the accumulation of membrane-permeating fluorescent cationic dye tetramethylrhodamine ethyl ester (TMRE). Cell images were obtained with an Eclipse Ti-E microscope equipped with a confocal A1 module (Nikon Corporation, Japan) using the NIS-Elements software. TMRE fluorescence was excited with a laser at 532 nm and recorded at 570-620 nm.

**Reactive oxygen species.** ROS generation in pea plant cells was detected by the ROS-mediated oxidation of nonfluorescent 2',7'-dichlorofluorescein diacetate (DCFH-DA) into fluorescent 2',7'-dichlorofluorescein (DCF). DCF formation can involve ions of transient metals and cytochrome *c*, which has the activity similar to that of peroxidase [17, 18]. The epidermis fixed on a plastic plate with a silicone grease was placed into a cuvette with 2 ml of the incubation medium. DCFH-DA and other reagents were added to the cuvette, and DCF fluorescence was measured with a VersaFluor fluorimeter (Bio-Rad) at  $\lambda_{ex} = 485\text{--}495$  nm and  $\lambda_{em} = 515\text{--}525$  nm.

**Cells incubation with reagents.** The effects of chemical compounds on the pea plant cells were determined by placing leaf epidermis into the wells of polystyrene plates with 2 ml of bidistilled water, followed by the addition of reagents and incubation. In the control, reagents were not added. Incubation conditions are described in the legends to the figures.

**Cell viability assay.** The death of pea cells was determined from the destruction of cell nuclei [19]. After incubation, the epidermis was treated for 5 min with the Battaglia fixative (a mixture of chloroform, 96% ethanol, glacial acetic acid and 40% formalin at a 5 : 5 : 1 : 1 ratio), washed in ethanol and water for 10 and 5 min, respectively, and stained with the nuclear dye Carazzi's hematoxylin for 1 h. Stained epidermis was washed with water and examined with a Primo Star light microscope (Carl Zeiss, Germany). The content of cells without nuclei and

cells with destructed nuclei was determined for each experimental variant.

**Cell membrane integrity assay.** The permeability of the cell plasma membrane was determined using the fluorescent dye propidium iodide (PI), which cannot pass through an intact membrane and binds to the nuclear DNA in the cells with damaged membranes [20]. After incubation with the tested reagents, the epidermis was stained with 2  $\mu\text{M}$  PI for 20 min and examined with an Axiovert 200M fluorescence microscope (Carl Zeiss) at  $\lambda_{\text{ex}} = 525\text{--}565$  nm and  $\lambda_{\text{em}} = 575\text{--}640$  nm, and the proportion of cells with the PI-stained nuclei (i.e., with the permeable plasma membrane) was calculated.

**Statistical analysis.** The number of cells with the damaged nuclei or permeable membrane was determined in a sample of 300–600 cells from 2–3 epidermal films in each experimental variant; each experiment was repeated 2–3 times. The figures show the representative recordings and images of the experiments. The data in the figures are presented as mean  $\pm$  95% confidence intervals (significance level  $\alpha = 0.05$ ).

## RESULTS AND DISCUSSION

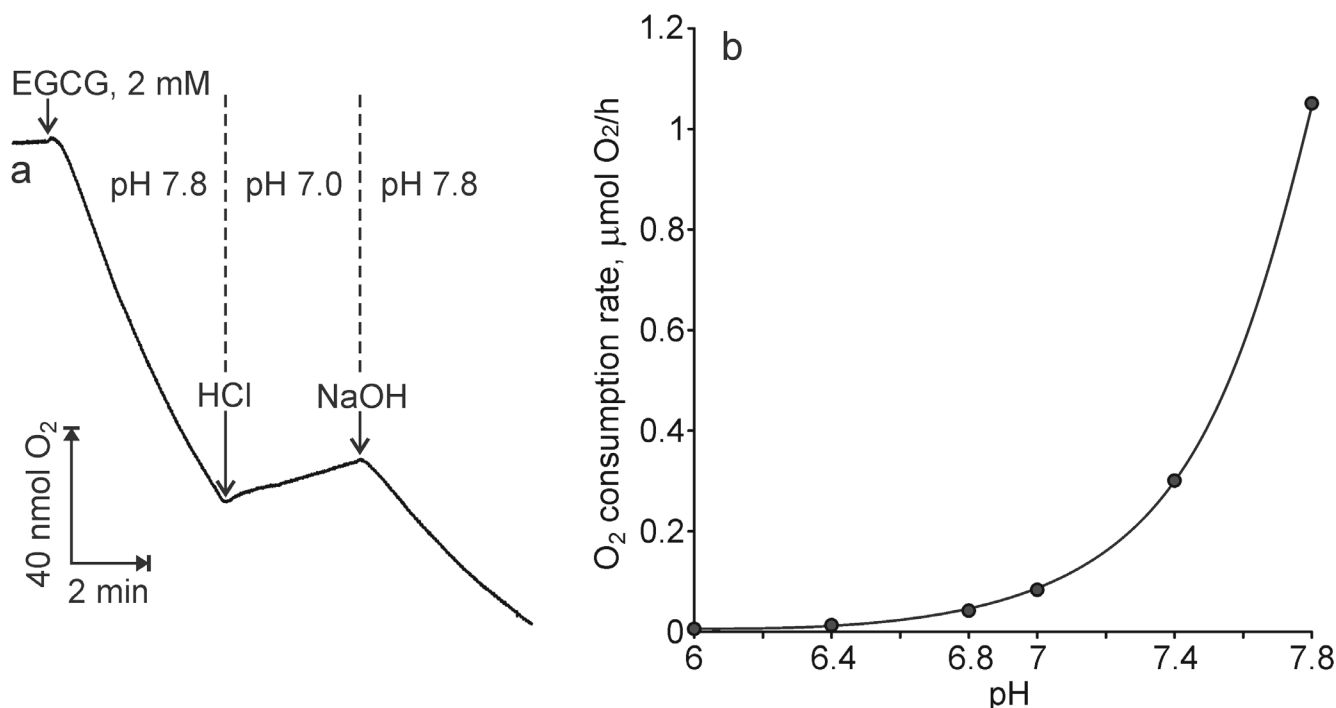
**EGCG effect *in vitro*.** EGCG was oxidized in a buffer solution at pH 7.8 (Fig. 1a). The  $\text{O}_2$  consumption ceased when the pH value was decreased to 7.0 by addition of hydrochloric acid and restarted with the following addition of an alkali to pH 7.8. The rate of EGCG oxida-

tion depended on the incubation medium, increasing or decreasing at weakly alkaline or weakly acidic pH values, respectively (Fig. 1b).

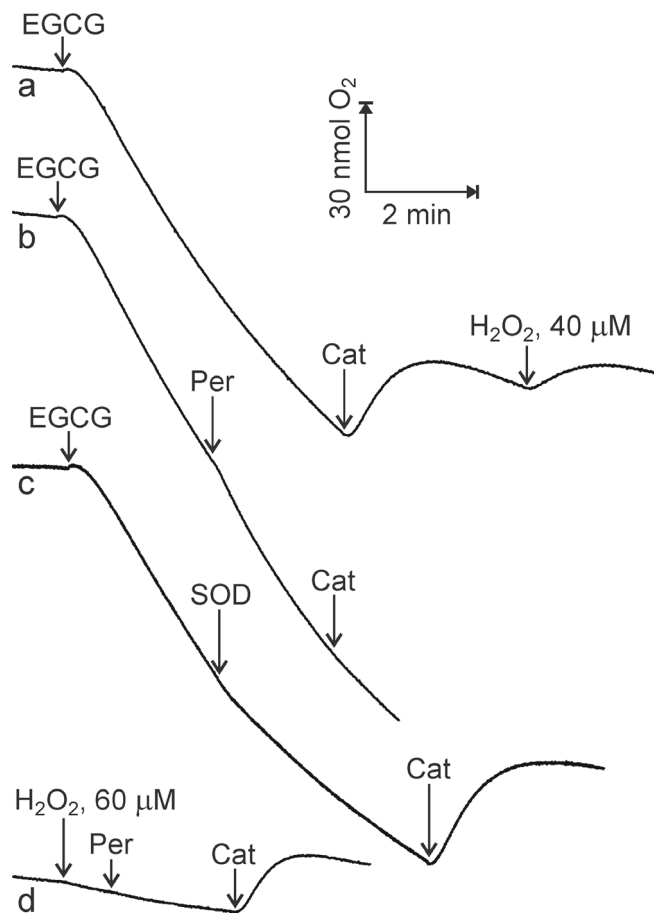
Adding catalase during EGCG oxidation (pH 7.8) caused the release of  $\text{O}_2$ , indicating  $\text{H}_2\text{O}_2$  formation (Fig. 2a).  $\text{O}_2$  was also released upon subsequent addition of  $\text{H}_2\text{O}_2$  to the incubation medium. Horseradish peroxidase added before catalase prevented the release of  $\text{O}_2$  (Fig. 2b). Superoxide dismutase reduced the rate of  $\text{O}_2$  consumption (Fig. 2c), which was due to the  $\text{O}_2$  production in the superoxide dismutase-catalyzed reaction and indicated formation of superoxide anion radical ( $\text{O}_2^-$ ) during EGCG oxidation. Incubation of  $\text{H}_2\text{O}_2$  with peroxidase in the absence of EGCG did not abolish  $\text{O}_2$  release after addition of catalase (Fig. 2d; cf. 2b).

$\text{H}_2\text{O}_2$  generation was detected with Amplex Red at pH 6.0, i.e., when EGCG oxidation was suppressed (Fig. 1b). The reaction between  $\text{H}_2\text{O}_2$ , peroxidase, and Amplex Red resulted in the formation of fluorescent resorufin (Fig. 3a).  $\text{H}_2\text{O}_2$  generation was observed after addition of peroxidase and NADH (irrespectively of the order of addition) (Fig. 3, b and c) as a result of peroxidase-oxidase reactions accompanied by the ROS ( $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ ) generation [21–24]. Catalase suppressed  $\text{H}_2\text{O}_2$  generation (Fig. 3b). EGCG at micromolar concentrations reduced the rate of  $\text{H}_2\text{O}_2$  production (Fig. 3c).

EGCG in combination with peroxidase suppressed  $\text{H}_2\text{O}_2$  generation (Fig. 3d) even in the incubation medium with pH 7.8, where EGCG oxidation took place (Fig. 1). The production of  $\text{H}_2\text{O}_2$  was higher and EGCG efficiency



**Fig. 1.** EGCG oxidation in the incubation medium (a) and dependence of the EGCG oxidation rate on the incubation medium pH (b). Incubation medium: 50 mM Tricine–KOH buffer (pH 7.8) with 35 mM NaCl and 1 mM  $\text{MgCl}_2$  (a) or 50 mM  $\text{KH}_2\text{PO}_4$ –NaOH buffer (pH 6–7.8) with 1 mM  $\text{MgCl}_2$  (b). Medium pH was changed by adding HCl or NaOH (a). Additions: 2 mM EGCG, HCl, NaOH.

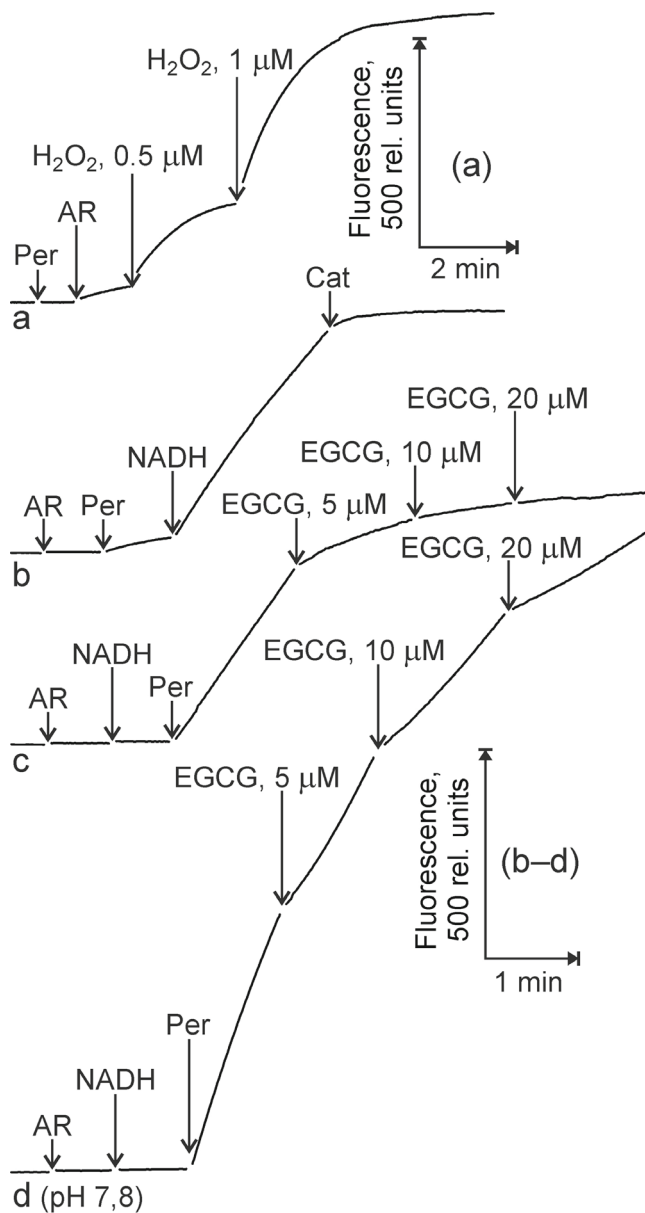


**Fig. 2.** The effects of catalase (Cat),  $\text{H}_2\text{O}_2$ , horseradish peroxidase (Per), and superoxide dismutase (SOD) on EGCG oxidation. Incubation medium: 50 mM Tricine-KOH buffer (pH 7.8) with 35 mM NaCl and 1 mM  $\text{MgCl}_2$ . Additions: 2 mM EGCG, 40 U/ml Cat,  $\text{H}_2\text{O}_2$ , 20 U/ml Per, 60 U/ml SOD.

was lower at pH 7.8 than at pH 6.0. EGCG at a concentration of 2 mM also prevented  $\text{H}_2\text{O}_2$  production at both pH 6.0 and 7.8 (data not shown).

Besides being oxidized in a weakly alkaline solution in the reaction consuming  $\text{O}_2$  (Fig. 1), EGCG generated  $\text{H}_2\text{O}_2$  (Fig. 2a), which was in agreement with the earlier published data. Thus, it was found that EGCG reduced  $\text{O}_2$  to  $\text{O}_2^-$ , resulting in the  $\text{H}_2\text{O}_2$  accumulation. EGCG oxidation was accompanied by the formation of EGCG dimers (theasinensins A and D), which were unstable and formed a number of chemical compounds, some of them cytotoxic [14]. At the same time, EGCG functioned as a peroxidase substrate (Fig. 2, b and d). In the presence of peroxidase, EGCG utilized  $\text{H}_2\text{O}_2$  that was produced as a result of autoxidation (Fig. 2) or peroxidase-oxidase reactions with NADH (Fig. 3). Hence, EGCG acts as either pro- or antioxidant depending on reaction conditions (pH, peroxidase activity).

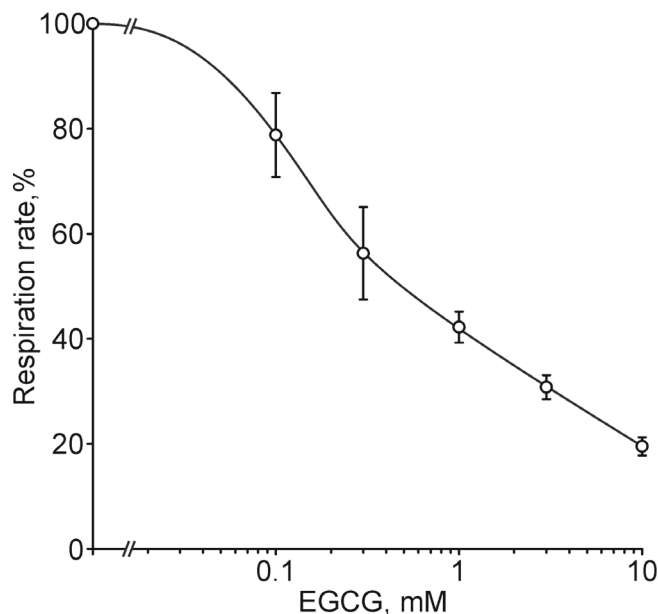
The range of pH values within which the rate of EGCG oxidation changed (Fig. 1b) was close to the physiological one. Measuring the pH value in different cell



**Fig. 3.** The effect of EGCG on the rate of fluorescent resorufin formation via nonfluorescent Amplex Red (AR) oxidation by horseradish peroxidase (Per). Incubation medium: 50 mM  $\text{KH}_2\text{PO}_4$ -NaOH buffer (pH 6.0) with 1 mM  $\text{MgCl}_2$  (a-c) or 50 mM Tricine-KOH buffer (pH 7.8) with 35 mM NaCl and 1 mM  $\text{MgCl}_2$  (d). Additions: 0.1 U/ml Per, 5  $\mu\text{M}$  AR,  $\text{H}_2\text{O}_2$ , 0.1 mM NADH, 40 U/ml catalase (Cat), EGCG.

compartments of human endothelial ECV304 cells using a pH-sensitive fluorescent protein showed pH 7.6 in the cytoplasm, pH 7.8 in the mitochondrial matrix; and pH 6.9 in the mitochondrial intermembrane space [25]. Therefore, the rates of EGCG oxidation and associated  $\text{H}_2\text{O}_2$  production can vary between different cell compartments.

**EGCG effects *in vivo*.** At a concentration of 0.1-10 mM, EGCG reduced the respiratory rate in pea leaf cuttings (Fig. 4), as it was determined from the  $\text{O}_2$  consumption.



**Fig. 4.** The effect of EGCG on the respiration in pea leaf cuttings. Incubation medium: 50 mM  $\text{KH}_2\text{PO}_4$ –NaOH buffer (pH 6.0) with 1 mM  $\text{MgCl}_2$ . The initial respiratory rate ( $70 \pm 15 \mu\text{mol O}_2/\text{h}$  per 1 g of wet weight) in leaf cuttings with no additions was taken as 100%.

The experiment was performed at pH 6.0, i.e., when EGCG oxidation was inhibited (Fig. 1b).

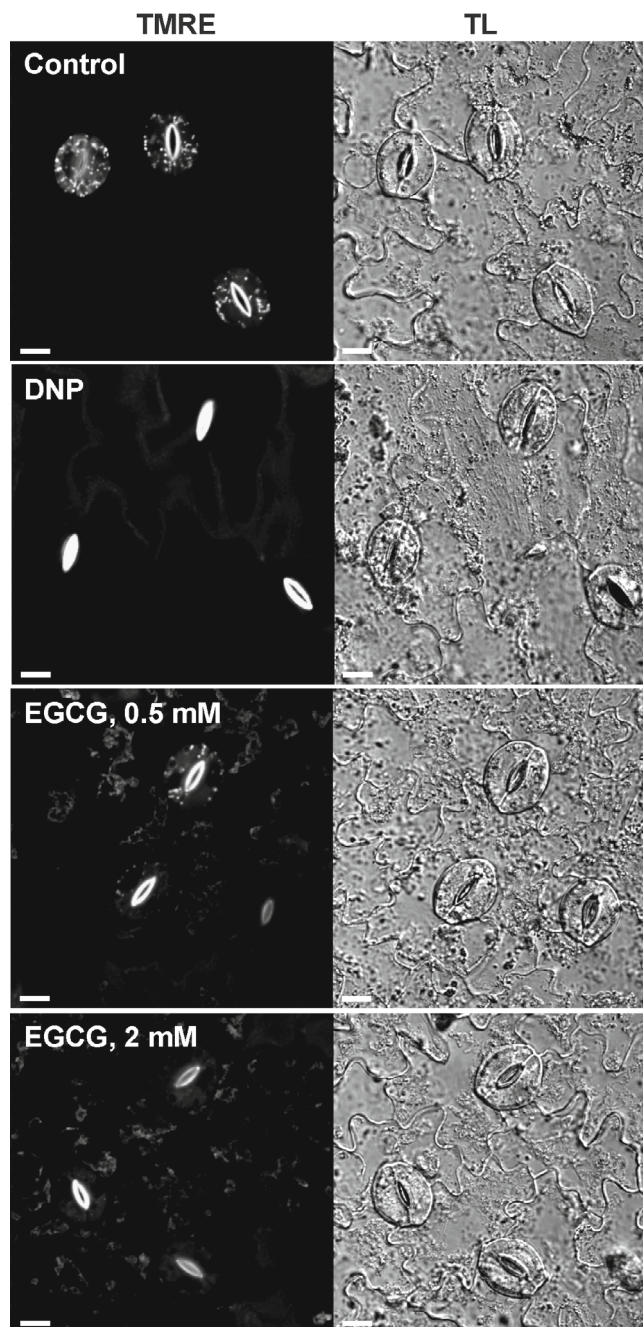
The published data on the effect of EGCG on the mitochondria are contradictory. In the mitochondria isolated from rat liver, EGCG was found to bound to the electron transport chain (ETC) complexes. Herewith EGCG at the concentrations below 0.1 mM reduced the activity of these complexes, especially complexes II and III, but by no more than 30%. The action of EGCG was more pronounced upon induction of the  $\text{Ca}^{2+}$ -dependent nonspecific permeability of the inner mitochondrial membrane, while cyclosporine A (inhibitor of the mitochondrial permeability transition pore) prevented this effect. Apparently, EGCG penetration into the mitochondria was limited by the lipid membrane [26].

On the contrary, EGCG restored the activity of the ETC complexes I, II, and IV impaired by cisplatin in mouse kidney cells due to its antioxidant effect [27]. EGCG at a concentration of 10  $\mu\text{M}$  accelerated respiration and increased the activity of cytochrome *c* oxidase in human neurons and astrocytes [28]. In the cells of human bone tissue, EGCG stimulated respiration [29].

The effect of EGCG on respiration can be due to the inhibition of glycolysis. According to the published data, EGCG reduces the activity of phosphoglycerate mutase [30]. EGCG was identified as the most efficient known inhibitor of phosphoglycerate mutase *in vitro* with  $\text{IC}_{50}$  of  $\sim 0.5 \mu\text{M}$ . *In vivo*, the effective concentrations of EGCG are 2-3 orders of magnitude higher, probably, due to the low permeability of membranes for this compound [30]. EGCG is more easily dissolved in DMSO

and ethanol than in water. In the cells, 85% of EGCG is found in the cytoplasm (where it has access to non-membrane glycolytic enzymes), about 2% – in the plasma membrane, and the rest of it – in the intracellular membrane fraction [31].

Figure 5 shows the pea leaf epidermis with the guard cells surrounded by the epidermal cells (see the images obtained in transmitted light). The epidermis was incubated



**Fig. 5.** The effect of 2,4-dinitrophenol (DNP) and EGCG on the TMRE accumulation in the mitochondria of guard cells in the leaf epidermis. Epidermal films in distilled water without additions (control), with 0.5 mM DNP or EGCG were stained with 0.5  $\mu\text{M}$  TMRE for 30 min. Scale bar, 10  $\mu\text{m}$ ; TMRE, TMRE fluorescence, TL, transmitted light.

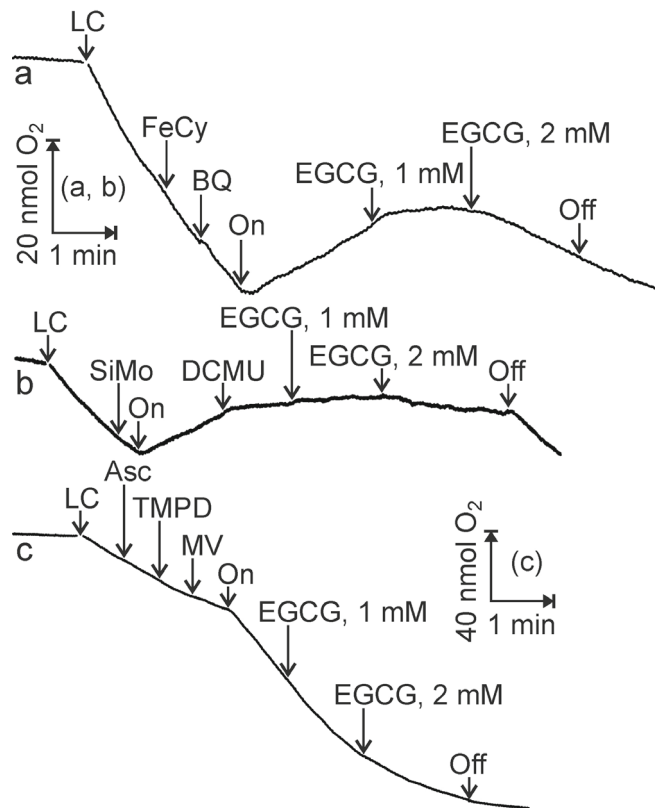
with the fluorescent dye TMRE, which accumulated the mitochondria of guard cells (the mitochondria of epidermal cells were in a different focal plane and, therefore, cannot be seen). The protonophore uncoupler 2,4-dinitrophenol (DNP), as well as 2 mM EGCG, prevented TMRE accumulation in the mitochondria. The treatment with 0.5 mM EGCG also inhibited TMRE accumulation in the mitochondria of guard cells but only partially (Fig. 5).

The EGCG-induced suppression of TMRE accumulation in the mitochondria (Fig. 5) could be associated with the inhibition of respiration (Fig. 4). An alternative explanation is that EGCG, similar to DNP, acted as an uncoupler. Thus, EGCG uncoupled oxidative phosphorylation in the rat hepatocytes mitochondria [32]. It is known that polyphenols, including EGCG, have the properties of weak protonophore uncouplers [33].

The effect of EGCG on the photosynthetic electron transport has been studied in the pea leaf cuttings in the incubation medium at pH 6.0.  $O_2$  was consumed in the dark in the process of respiration (Fig. 6). Ferricyanide and *p*-benzoquinone are electron acceptors. *p*-Benzoquinone, which is able to pass through the lipid membranes, reduces ferricyanide outside the cell and oxidizes plastoquinol, as well as the redox centers of Photosystem II and cytochrome *b<sub>6</sub>f* complex, which reduce plastoquinone in the chloroplasts. When the light was turned on in the presence of added electron acceptors, we observed the release of  $O_2$ , which was inhibited by the addition of 1 mM EGCG (Fig. 6a). Subsequent EGCG addition to the concentration of 2 mM completely suppressed photosynthetic production of  $O_2$ . After that, turning off the light had no effect.

Figure 6b shows the transport of electrons from  $H_2O$  (with the release of oxygen) via the Photosystem II redox centers to silicomolybdate in the light. Diuron, an inhibitor of electron transport from  $Q_A$  to  $Q_B$ , only partially inhibited  $O_2$  release, because silicomolybdate was reduced mainly by  $Q_A$  [34]. EGCG was less efficient at this fragment of the ETC and only partially inhibited the electron transport. EGCG at a concentration of 1 mM had no effect, while 2 mM EGCG converted slow  $O_2$  release to its slow consumption. Turning off the light significantly accelerated  $O_2$  consumption (Fig. 6b).

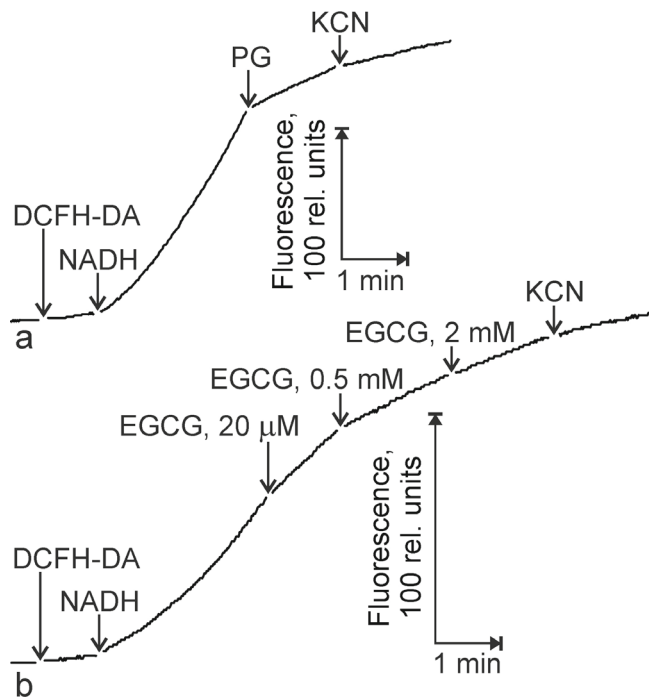
Illumination induced electron transport along the following pathway: ascorbate → *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMDP,  $E_0' = 0.26$  V) → P700 ( $E_0' = 0.45$  V) → redox centers of Photosystem I →  $Fe_4S_4$  cluster of  $F_B$  → methyl viologen →  $O_2$  (Fig. 6c). TMDP can also reduce plastocyanin ( $E_0' = 0.37$  V), but does not interact with the cytochrome *b<sub>6</sub>f* complex [35, 36]. Oxygen is consumed in the  $O_2^-$  production; therefore, its consumption increased when the light was turned on. The effect of EGCG at a concentration of 1 mM was insignificant; subsequent addition of 2 mM EGCG inhibited oxygen consumption (Fig. 6c).



**Fig. 6.** The effect of EGCG on the  $O_2$  release during electron transport from  $H_2O$  to *p*-benzoquinone (BQ) and ferricyanide (FeCy) (a) or to silicomolybdate (SiMo) in Photosystem II (b) and effect of EGCG on the  $O_2$  consumption during electron transport from ascorbate (Asc) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) to methyl viologen (MV) in Photosystem I (c) in pea leaf cuttings (LC) in the light. Incubation medium: 50 mM  $KH_2PO_4$ -NaOH buffer (pH 6.0) with 1 mM  $MgCl_2$ . Additions: 20 mg LC, 1 mM FeCy, 0.1 mM BQ, EGCG, 0.2 mM SiMo, 10  $\mu$ M diuron (DCMU), 1 mM Asc, 0.1 mM TMPD, 1 mM MV. On and Off, light on and off, respectively.

Our results demonstrated that EGCG inhibited electron transport in the photosynthetic ETC in chloroplasts (Fig. 6). There are no published data on the EGCG effect on photosynthesis in plants; however, it has been shown that EGCG inhibits photosynthetic reactions in the cyanobacterium *Microcystis aeruginosa* [37]. The efficiency of EGCG varied depending on the involved ETC components: Photosystem II → plastoquinone pool → cytochrome *b<sub>6</sub>f* complex (*p*-benzoquinone) > Photosystem I (TMPD and methyl viologen) > Photosystem II (silicomolybdate).

Based on the  $E_0'$  values, it can be assumed that EGCG ( $E_0' = 0.1$ -0.15 V) [38] reduces the Rieske iron-sulfur protein (0.3-0.32 V) and cytochrome *f* (0.35-0.38 V) in the cytochrome *b<sub>6</sub>f* complex [39] and oxidizes the FeS clusters (from -0.7 to -0.55 V) of Photosystem I, while reduction of chloroplast plastoquinone (from -0.13 to 0.1 V), especially  $Q_A$  (-0.13 V) and  $Q_B$  (-0.01 V) quinones in Photosystem II, is less probable. Apparently, the inhibitory effect of EGCG on photosynthesis is

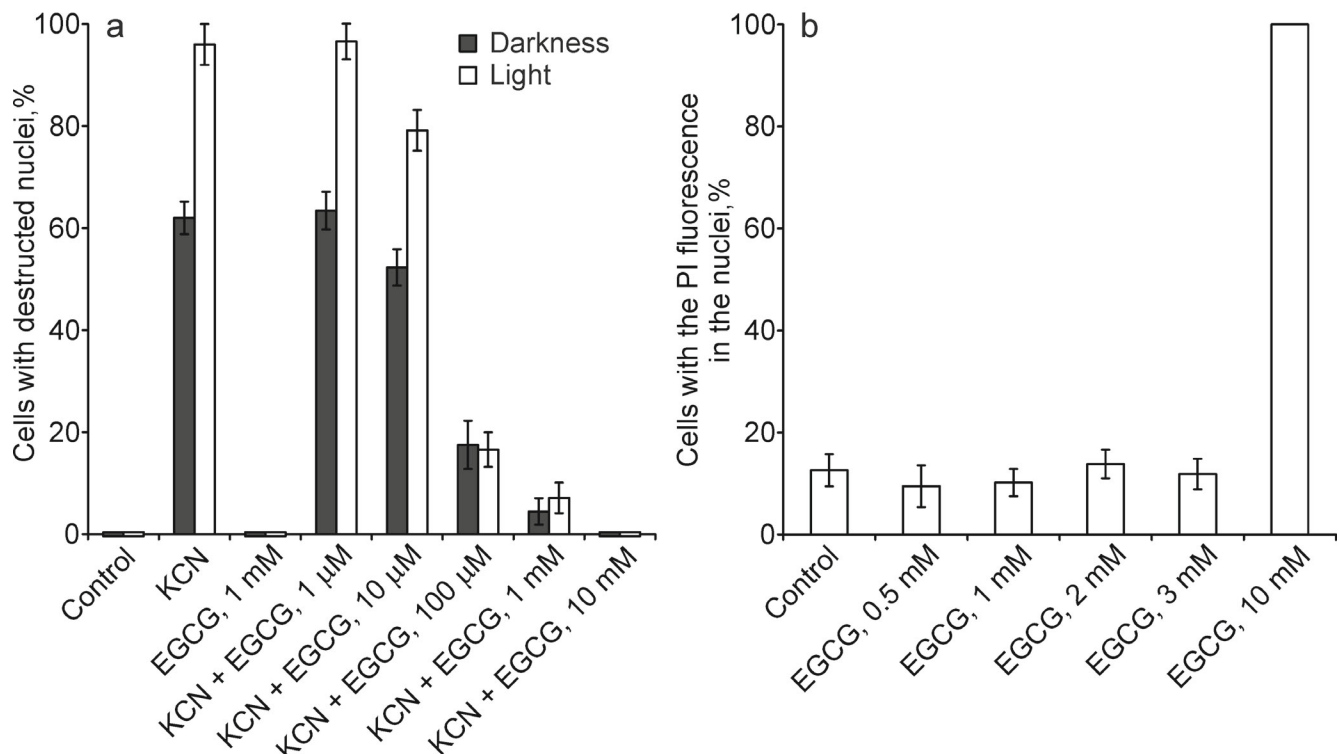


**Fig. 7.** The effect of EGCG on the rate of fluorescent DCF formation from nonfluorescent DCFH-DA in the pea leaf epidermis. The epidermis was placed into a cuvette with the following incubation medium: 50 mM  $\text{KH}_2\text{PO}_4$ -NaOH buffer (pH 6.0) with 1 mM  $\text{MgCl}_2$ . Additions: 20  $\mu\text{M}$  DCFH-DA, 1 mM NADH, 0.5 mM propyl gallate (PG), 2.5 mM KCN, EGCG.

determined by the redox reactions with the components of photosynthetic ETC. This assumption is in agreement with the fact that EGCG inhibits electron transport more efficiently in the cytochrome  $b_6f$  complex and Photosystem I than in Photosystem II (Fig. 6).

The level of ROS in the pea leaf epidermis was determined by the fluorescence of DCF formed from non-fluorescent DCFH-DA. Addition of NADH induced ROS generation, thus increasing the quantum yield of DCF fluorescence (Fig. 7). ROS were produced as a result of peroxidase-oxidase reactions in the apoplast (because cell membrane is impermeable for NADH). The antioxidant propyl gallate and KCN, an inhibitor of heme-containing enzymes (including peroxidases), decreased the DCF fluorescence (Fig. 7a). EGCG exhibited the antioxidant properties and decreased the rate of DCF formation by 30 and 70% when 20  $\mu\text{M}$  and 2 mM were added, respectively (Fig. 7b).

KCN, a PCD inducer, caused the death of guard cells, which was registered from the destruction of cell nuclei (Fig. 8a). KCN was more efficient in the light than in the dark, since chloroplasts contribute to PCD in plants [19]. EGCG at a concentration of 1  $\mu\text{M}$  had no effect on the damage of guard cell nuclei. EGCG at concentrations from 10  $\mu\text{M}$  to 10 mM reduced the KCN-induced destruction of the nuclei in the dark and in the light (Fig. 8a). It is unlikely that the effect of EGCG was



**Fig. 8.** The effect of EGCG on the KCN-induced destruction of cell nuclei (a) and on the plasma membrane permeability for PI (b) in guard cells of the pea leaf epidermis. a) After addition of 2.5 mM KCN and EGCG, the epidermis was incubated for 20 h in the dark or in the light, stained with hematoxylin, and assessed for the number of cells with destroyed nuclei. b) After addition of EGCG, the epidermis was incubated for 2 h in the dark, stained with PI, and assessed for the number of cells with PI fluorescence in cell nuclei.

related to its influence on the peroxidase activity, respiration, or photosynthesis, because KCN *per se* is an efficient inhibitor of peroxidase reaction, respiration, and photosynthesis. However, the death of guard cells caused by the treatment with KCN was sensitive to antioxidants [19], and EGCG may exhibit its antioxidant activity not only in combination with peroxidase, but also due to the presence of gallic acid residues in its structure [24].

The KCN-induced destruction of the guard cell nuclei may be suppressed as a result of switching from PCD to unprogrammed necrosis, e.g., upon the use of detergents or increase in the concentration of PCD inducers [40]. Therefore, the effect of EGCG on the barrier function of the plasma membrane was tested. EGCG at a concentration of 0.5–3 mM had no effect on the plasma membrane permeability for PI, while 10 mM EGCG increased it (Fig. 8b). Therefore, the reduction in the cyanide-induced destruction of nuclei after the treatment with EGCG (Fig. 8a) can be associated with the plasma membrane damage only at the high EGCG concentration (10 mM), but not at the concentrations from 10  $\mu$ M to 1 mM.

The prooxidant properties of EGCG can explain its effects, such as induction of the plasma membrane permeability,  $\Delta\psi$  dissipation in the mitochondria, and inhibition of respiration and photosynthesis. Inside the cells, at slightly alkaline pH values, EGCG is oxidized with the formation of H<sub>2</sub>O<sub>2</sub>. Hence, the highest rate of EGCG oxidation should be expected in the chloroplast stroma (pH up to 8.0) in the light [41] and in the mitochondrial matrix (pH 7.8) [25]. H<sub>2</sub>O<sub>2</sub> can change the physicochemical properties of cell membranes, induce mitochondrial membrane depolarization, as well as lipid peroxidation and impair membrane permeability barrier [42–44].

In conclusion, the redox properties of EGCG in solution and its effect on plant cells have been studied. EGCG *in vitro* was oxidized by oxygen at physiological pH values. The decrease in pH slowed down this reaction. EGCG oxidation was accompanied by the production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. At the same time, EGCG functioned as an electron donor for peroxidase and utilized H<sub>2</sub>O<sub>2</sub> in combination with this enzyme. We also investigated the effect of EGCG on the energy metabolism and death of plant cells. At the concentrations of 0.5–3 mM, EGCG inhibited respiration in pea cells, reduced  $\Delta\psi$  in the mitochondria, and inhibited electron transport in the photosynthetic ETC. EGCG also decreased ROS generation that was caused by the addition of NADH to the cells. At the concentrations from 10  $\mu$ M to 1 mM, EGCG prevented the KCN-induced PCD. At higher concentrations (10 mM), EGCG induced the permeability of the cell plasma membrane for PI. Generally, in spite of the prooxidant properties of EGCG, its cytoprotective effect is manifested at the concentrations below 1 mM.

The antioxidant effect of EGCG on plant cells was stronger than its prooxidant effect. Similar properties of

EGCG have been reported in most studies conducted in animal models. EGCG is presumably oxidized at different rates in various cell compartments, depending on pH value. Because of its properties, in particular, the anti- and prooxidant activities, EGCG can be a promising agent for biological research and plant protection. However, one should take into account its inhibitory effect on respiration and photosynthesis, as well as limitations associated with the use of high EGCG concentrations causing the cell membrane damage.

**Contributions.** V.D. Samuilov developed the concept, supervised the study, and edited the manuscript; O.V. Samuilova discussed the results and edited the manuscript; D.B. Kiselevsky performed the experiments and wrote the manuscript.

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