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RESEARCH ARTICLE

The Effect of Neurotransmitters on Programmed Cell Death and Formation of Reactive Oxygen Species in the Pea Leaf Epidermis

D. B. Kiselevsky[a,*] (ORCID: 0000-0001-6050-2940), A. V. Oleskin[b] (ORCID: 0000-0002-6816-1615), and V. D. Samuilov[a]

[a] *Department of Immunology, Faculty of Biology, Moscow State University, Moscow, 119234 Russia*

[b] *Department of General Ecology and Hydrobiology, Faculty of Biology, Moscow State University, Moscow, 119234 Russia*

**e-mail: dkiselevs@mail.ru*

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Abstract—Neurotransmitters are found not only in animals, but also in other living organisms, including plants. However, the data on the functions of these compounds in the plant world are far from being comprehensive. In particular, the issue concerning their impact on plant cell death still demands further research. In the present study, we tested the effects of neurotransmitters on programmed cell death and the formation of reactive oxygen species (ROS) in plants. Programmed cell death was estimated from the destruction of cell nuclei. ROS was determined using 2',7'-dichlorofluorescein. Dopamine, norepinephrine, serotonin, histamine, acetylcholine and acetylthiocholine (its synthetic analog) were used. The catecholamines dopamine and norepinephrine suppressed KCN-induced destruction of guard cell nuclei in the pea leaf epidermis at concentrations of 0.01–1 mM. In contrast, serotonin and acetylcholine (1–3 mM) promoted the destruction of nuclei that was induced by KCN. Histamine and acetylthiocholine had no effect on KCN-induced destruction of nuclei at concentrations of 0.01–3 mM. Unlike natural neurotransmitters, acetylthiocholine (3 mM), caused the destruction of guard cell nuclei even when KCN was absent. Dopamine, norepinephrine, and serotonin reduced menadione-induced ROS formation in the pea leaf epidermis. No similar effect was observed with histamine, acetylcholine, and acetylthiocholine. Therefore, dopamine, norepinephrine, and serotonin possess antioxidant properties in plants. In addition, dopamine and norepinephrine prevent cell death.

Keywords: neurotransmitters, biogenic amines, programmed cell death, reactive oxygen species, pea

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INTRODUCTION

Neurotransmitters are low-molecular compounds of various chemical nature; their important representatives are biogenic amines (dopamine, norepinephrine, adrenaline, serotonin, histamine, etc.) and acetylcholine [1]. Their most well-known function in animals is involvement in the excitation transfer from one cell to another. Neurotransmitters also perform other functions. For example, histamine plays an important role in immunoregulation and allergic response; it is also involved in the regulation of cell proliferation and differentiation, hematopoiesis, embryonic development, and regeneration [2].

These compounds are found not only in animals, but also in other organisms, including plants. The content of neurotransmitters in plants varies depending on the species and tissue; generally, it is comparable to that in animals [1].

The functions of neurotransmitters in plants are not associated with the transmission of nerve impulses; therefore, it is more appropriate to call them "biomediators" when referring to plants [1]. These compounds are involved in organ formation, flowering, ion transport, photosynthesis, circadian rhythms, fruit ripening, photomorphogenesis (a light-dependent development process), and plant adaptation to environmental changes. Biogenic amines such as catecholamines have been now found in 28 species from 18 plant families tested [3]. Of note are increased amounts of dopamine (1–4 mg/g wet weight) found in flowers and fruits exemplified by *Araceae* inflorescences and banana pulp [4].

Biogenic amines (in particular, catecholamines) act as stress response factors. When potato leaves are damaged, the concentration of dopamine, norepinephrine and epinephrine increase significantly. The

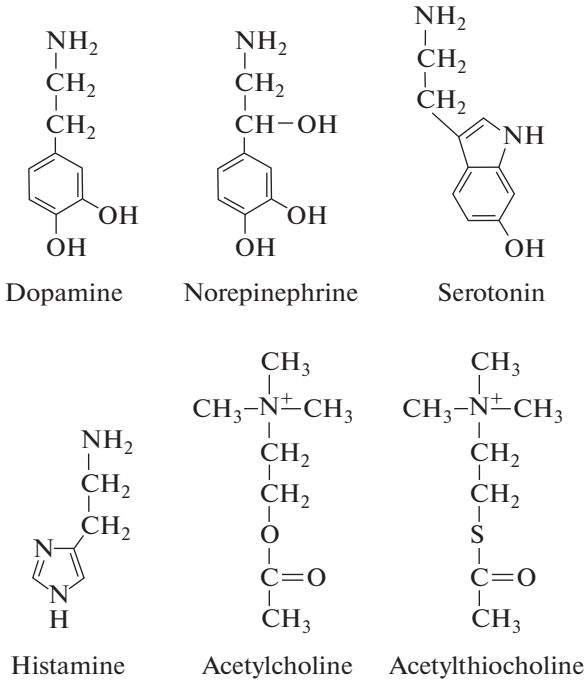

Fig. 1. Molecular structure of biomediators applied in the study.

dopamine content also increases in damaged callus of *Portulacca* [4].

Serotonin and its derivative melatonin are also important regulators of plant development at all stages, from seed germination to flowering [5]. Serotonin accelerates shoot growth in *Mimosa pudica* and *Hippeastrum hybridum*; in the latter, it also promotes seed germination. In rice plants, serotonin delays the aging process. Serotonin also plays a regulatory role in the formation of plant root tips. Serotonin and melatonin interact in plant tissues with such important regulatory compounds as nitric oxide and salicylic and jasmonic acids [6].

Acetylcholine is synthesized by at least 65 species of flowering plants belonging to 33 different families. This compound is present at high concentrations in nettle urticating hairs [3]. Addition of exogenous acetylcholine stimulates the growth of flowering plants (tomatoes, wheat, cowpeas, radish, bamboo buds), promotes their flowering, accelerates the movement of stomata, enhances the action of the phytochrome system, reduces the formation of the gaseous phytohormone ethylene, and prevents leaf curling. At the same time, the addition of acetylcholine to the cultivation medium suppresses the growth of root hairs in *Arabidopsis thaliana* [6].

Despite the data presented above, there is no published information about the effect of biomediators on the plant cell death. The present study was conducted to fill this gap.

The two different types of animal cell death are non-programmed (necrosis) and regulated or programmed cell death (PCD). PCD includes apoptosis, autophagy, pyroptosis, necroptosis, ferroptosis, netosis, and other cell death scenarios [7]. Plants are characterized by special forms of PCD, which are similar to apoptosis, necroptosis, and pyroptosis [8]. PCD in plants occurs during the development of reproductive organs, the formation of special tissues (conducting vessels and the aerenchyma), the formation and fall of leaves, and it forms a part of a plant's immune response to pathogen, i.e., of a hypersensitive response [9, 10]. Cell death is subject to regulation by various small molecular compounds. Energy supply and reactive oxygen species (ROS) availability play an important role in the PCD process in plants [11]. For instance, antioxidants and energy metabolism-affecting agents or conditions exert an influence on cell death [12].

In our study, the biogenic amines dopamine and norepinephrine (catecholamines), serotonin, histamine, acetylcholine and its synthetic analogue acetylthiocholine (Fig. 1) were utilized. The study aimed to test the effects of these biomediators on PCD and ROS formation in plants.

MATERIALS AND METHODS

Research Subject

Experiments were carried out on epidermis isolated from the lower surface of leaves of 13–24-day-old pea seedlings (*Pisum sativum* L. cultivar Alpha). The epidermis was a monolayer of the following two types of cells: the guard cells of the stomata and the bulk cells of the epidermis (epidermal cells). Unlike guard cells, epidermal cells did not photosynthesize because they did not contain chloroplasts. Pea plants were grown at 18–26°C under periodic lighting conditions (light/dark, 16h/8h). They were illuminated with a DRiZ Reflux metal-halide lamp (Reflaks-S, Russia; 250 W) at a light intensity of $\sim$100 $\mu E\ m^{-2}\ s^{-1}$. The light intensity was measured with a Quantitherm PAR/Temp sensor (Hansatech, UK). Epidermal films were separated from the lower surface of leaves using tweezers.

Cell Death

PCD was recorded by the destruction of cell nuclei [12]. Epidermal films were placed in the wells of 6- or 12-well polystyrene culture plates (Greiner Bio-One, Austria) with 2 mL double-distilled water. Thereupon, the tested compounds were added, and the plates with the plant material were incubated for 22–24 hours. The composition and concentration of added compounds are indicated at Figs. 2–3 and their captions. The control plates were incubated without additions.

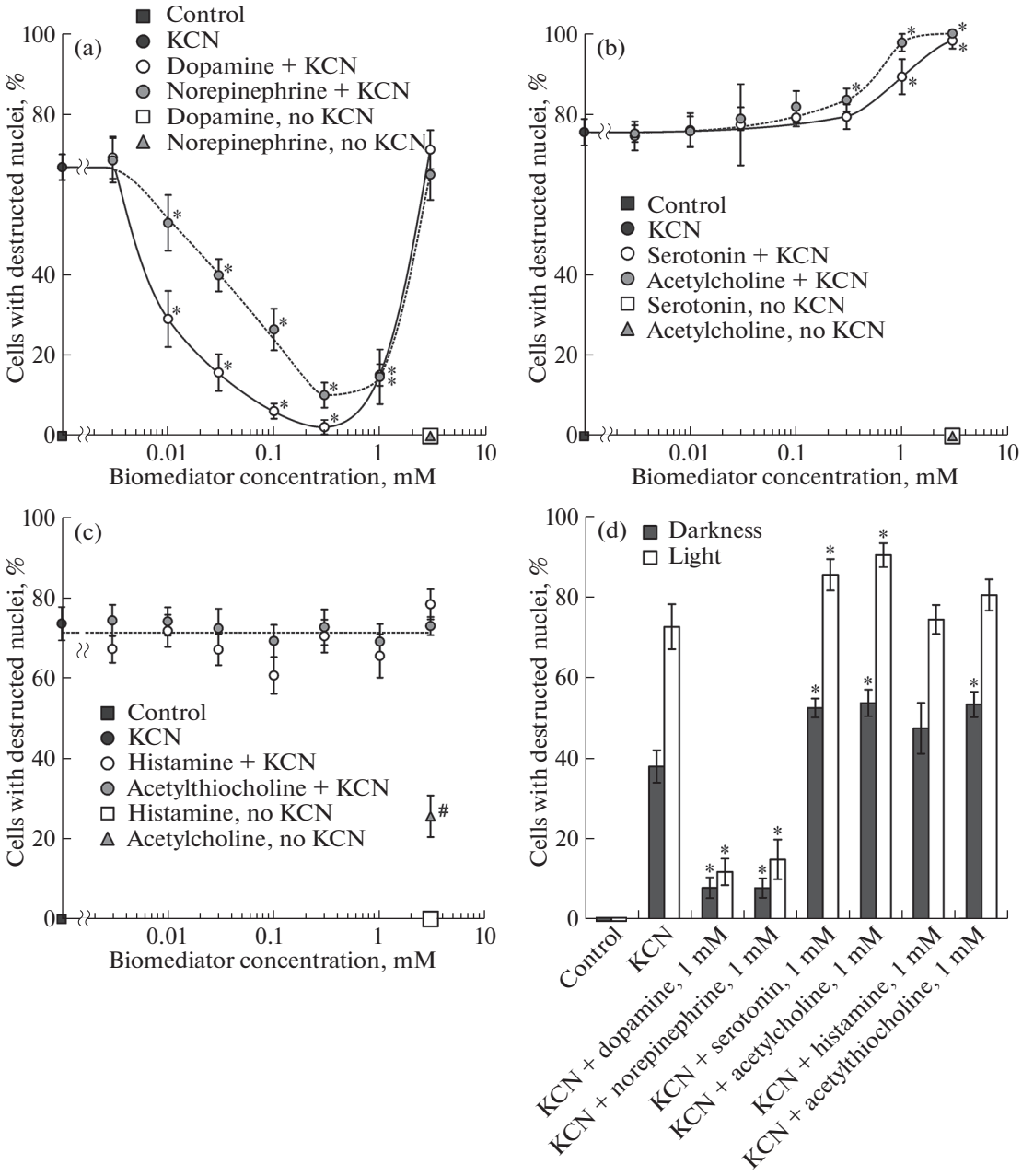

Fig. 2. Effect of biomediators on KCN-induced destruction of guard cell nuclei in the pea leaf epidermis. Biomediators and KCN (2.5 mM) were added to epidermis and incubated in the light (a–d) or in the dark (d). Significant differences ($p < 0.01$) between treatments are marked as follows: (#) "control" (no addition) vs "biomediator, no KCN"; (*) "KCN" vs "biomediator + KCN" combinations.

The experimental design is given in the legends to Figs. 2–3.

After incubation, the leaf epidermis was treated for 5 min with Battaglia fixative (a mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formaldehyde at a ratio of 5:5:1:1), washed for 10 min in ethanol and, thereupon, for 5 min in water to remove the fixative, and stained for 40 min with Carazzi's hematoxylin. The stained epidermal films were washed in water and examined using a Primo Star light microscope (Carl Zeiss, Germany). In each experimental treatment, the share of guard cells with destroyed nuclei (nuclei-deprived) was determined.

Formation of Reactive Oxygen Species

ROS in cells were determined from the fluorescence of 2',7'-dichlorofluorescein (DCF) that formed from non-fluorescent 2',7'-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA penetrates into cells and is

deacetylated by intracellular esterases and oxidized by ROS, mainly H_2O_2, with the participation of peroxidases, yielding fluorescent DCF [13]. It is also oxidized by hydroxyl radical ($OH^{\bullet}$) and other free radical compounds, but not by superoxide anion radical ($O_2^{-\bullet}$) [14]. The formation of DCF may involve metal ions of variable valence and cytochrome *c,* which exhibts a peroxidase-like activity [15].

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) was used as an agent to induce ROS production in cells. It is reduced by various enzymes that carry out redox reactions, including the components of the respiratory and photosynthetic electron transport chains. Menadione is oxidized by oxygen to $O_2^{-\bullet}$, which then produces H_2O_2 in a dismutation reaction [16–18].

The incubation medium (2 mL) added to a 4-mL spectrophotometric polystyrene cuvette contained 50 mM buffer Tricine-KOH (pH 7.8) with 0.4 M sucrose, 35 mM NaCl, and 1 mM $MgCl_2$. Silicagel was applied to glue the epidermis to the polystyrene plate. The plate with the epidermis was placed vertically in a cuvette with the incubation medium so that the angle between the plate plane and the cuvette walls was 45°. DCFH-DA, menadione, and other compounds were added sequentially to the incubation medium. DCF fluorescence was excited with light at 485–495 nm and recorded at 515–525 nm using a VersaFluor fluorimeter (Bio-Rad, USA) and the FluoSpectr 1.2 computer program (Lomonosov Moscow State University, Russia).

Statistical Data Processing

In experiments with cell death (microscopy), 300–750 cells were examined in 2–3 films of leaf epidermis for each experimental treatment and in control. The graphs show mean values ± 95% confidence intervals. In order to find out whether the differences in the average values of the samples obtained (in the control or KCN-treated system vs. the experimental systems) were significant, Student's t-test was applied at $p = 0.01$. ROS determination expreiments were repeated 2–3 times. The results of typical experiments are presented.

RESULTS AND DISCUSSION

The effect of biomediators, functioning as neurotransmitters in the nervous system of animals, on the programmed death of pea guard cells was determined from the destruction of cell nuclei. KCN was used as a cell death inducer [12]. In plant cells, KCN inhibits cytochrome *c* oxidase in mitochondria, ribulose bisphosphate carboxylase in chloroplasts [19], and enzymes that utilize ROS, including catalase, peroxidases, and Cu, Zn superoxide dismutase [20]. KCN causes PCD, which is manifests itself in the following symptoms: destruction and fragmentation of cell nuclei, condensation of nuclear chromatin, internucleosome fragmentation of DNA, as well as sensitivity to antioxidants, protein synthesis inhibitors, and factors affecting energy metabolism [12, 21, 22].

Treating epidermis with KCN under illumination during the day caused the destruction of nuclei in 60–80% of guard cells (Fig. 2). In the dark, KCN destroyed the nuclei in about 40% of cells (Fig. 2c). Dopamine and norepinephrine at concentrations of 0.01–1 mM suppressed KCN-induced destruction of guard cell nuclei in the light (Fig. 2a), but the protective effect was abolished if their concentrations increased to 3 mM. In contrast, 1–3 mM serotonin and acetylcholine enhanced KCN-caused nuclear destruction under illumination (Fig. 2b). Histamine and acetylthiocholine had no effect on KCN-dependent nuclear destruction within the tested concentration range (Fig. 2c). When incubated in the dark, the biomediators acted in a similar fashion: dopamine and norepinephrine suppressed nuclear destruction that was caused by KCN, and other tested compounds did not produce any similar effect (Fig. 2d).

The issue to raise is whether biomediators can react chemically with cyanide in our experiments, thereby affecting the results. Our data seem to militate against this suggestion. Hydrocyanic acid formylates phenolic compounds in the Gattermann reaction; however, this requires HCl and certain metal chlorides as catalysts. According to our data (Fig. 2), adding biomediators at a concentration of 3 mM, close to KCN concentration (2.5 mM), did not prevent the destruction of nuclei, although this would be expected if KCN was consumed as a result of its chemical interaction with biomediators. Importantly, the effect of cyanide against the background of equimolar concentrations of epinephrine, norepinephrine, and serotonin was previously demonstrated in experiments on human umbilical veins and arteries [23].

Among the biomediators tested, only acetylthiocholine caused nuclear destruction per se without KCN addition (Fig. 2c), in contrast to its natural analogue acetylcholine. Plausibly, plant cell death could be brought about by thiocholine, a product of acetylthiocholine hydrolysis by cholinesterases. Cholinesterase activity is inherent in plants, especially belonging to the Fabaceae family [1] that includes peas. Thiocholine is a thiol known to induce apoptosis in mammalian cells [24]. Acetylthiocholine in combination with KCN did not enhance nuclear destruction in our experiments (Fig. 2c), which may be due to the inhibitory effect of cyanide on the activity of cholinesterases.

The effect of biomediators on the ROS formation in pea leaf epidermis was also investigated. It was determined from DCF fluorescence after adding DCFH-DA to the incubation medium (Fig. 3).

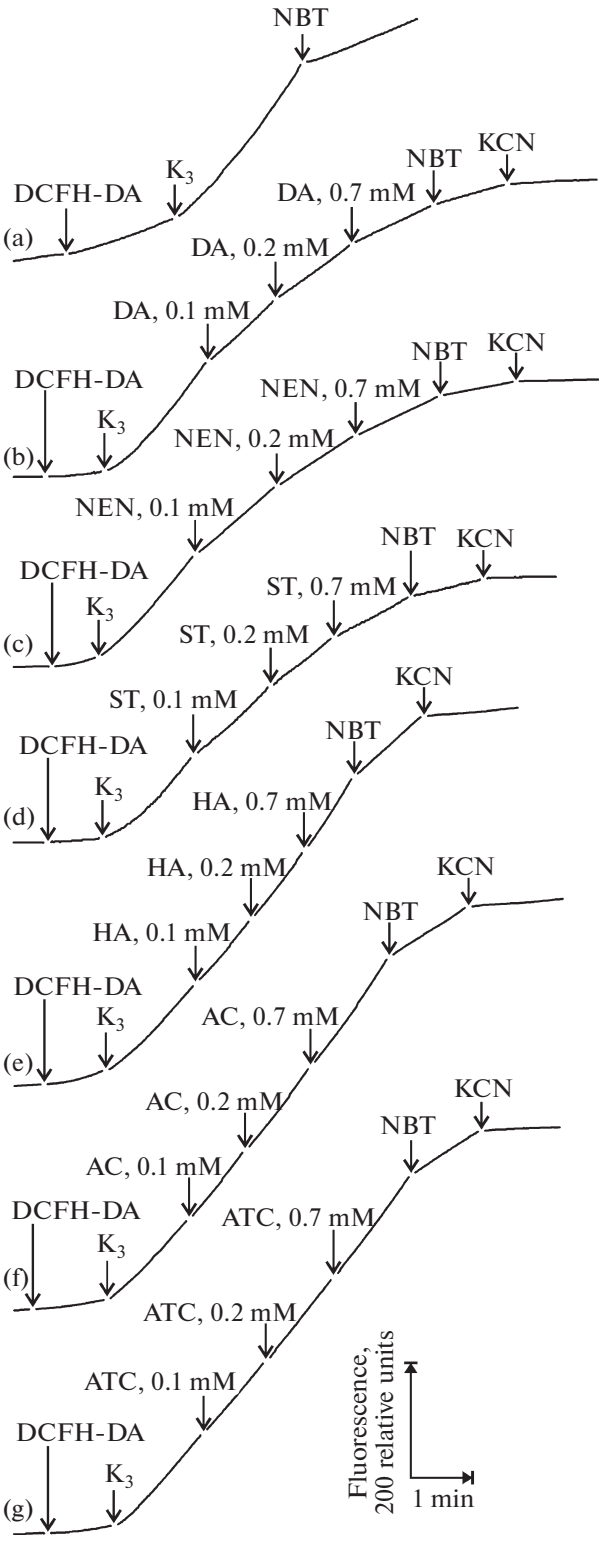

Fig. 3. Effect of dopamine (DA), norepinephrine (NEN), serotonin (ST), histamine (HA), acetylcholine (AC), and acetylthiocholine (ATC) on the rate of synthesis of fluorescent DCF from non-fluorescent DCFH-DA in epidermal pea leaf films. Additives: 20 μM DCFH-DA, 0.1 mM menadione (K_3), 0.2 mM nitro blue tetrazolium (NBT), DA/NEN/ST/HA/AC or ATC, and 2.5 mM KCN.

Menadione stimulated ROS generation, increasing the fluorescent DCF production rate by 3.5 times (Fig. 3a). The effect of menadione was reduced by adding an antioxidant, nitro blue tetrazolium, which reacted with the superoxide anion radical ($O_2^{-\cdot}$) to form formazan [25].

Dopamine, norepinephrine, and serotonin suppressed ROS formation in epidermis (Fig. 3b–3d). Supplementation of dopamine, norepinephrine or serotonin (0.1 mM) reduced the DCF production rate by 28, 29 and 20%, respectively, compared to that after DCFH-DA and menadione addition (without biomediators). With 1 mM dopamine, norepinephrine, or serotonin in the incubation medium, the DCF production rate decreased more than twofold (by 65, 61, and 56%, respectively).

Histamine, acetylcholine, and acetylthiocholine failed to produce a similar effect (Fig. 3e–3g). On the contrary, histamine (1 mM) increased the DCF production rate in the epidermis by 37%, acetylcholine by 25%, and acetylthiocholine by 10%.

Nitro blue tetrazolium reduced the DCF production rate 1.7–2.5 fold against the background of all tested biomediators. Addition of KCN that functioned as a peroxidase inhibitor abolished the peroxidase activity-dependent DCF production (Fig. 3b–3g).

Antioxidant effects were exerted by phenolic biomediators with one or two hydroxyl groups associated with the aromatic ring in the molecule (Fig. 1). Dopamine and norepinephrine containing two OH groups protected plant cells from KCN-mediated death, unlike serotonin (Fig. 2a, 2b). Evidence was presented in the literature concerning the antioxidant properties of various phenolic compounds [26–28].

There are data on the antioxidant effect of phenolic biomediators (dopamine and norepinephrine) [29, 30]. In vitro, their antioxidant activity was comparable to that of another antioxidant, α-tocopherol (vitamin E). However, α-tocopherol was less efficient in utilizing superoxide anione radical than dopamine. The biogenic amine tyramine that contains only one hydroxyl group in the phenolic ring of the molecule, similar to serotonin, exhibited antioxidant properties [30]. Serotonin displays antioxidant effects both in vitro and in vivo [31, 32]. Dopamine, norepinephrine, and serotonin can be used as substrates for peroxidase oxidation that involves H_2O_2 [32, 33].

Nonetheless, biogenic amines may have pro-oxidant activity and cause oxidative stress, favoring the development of neurodegenerative diseases. By participating in the peroxidase reaction, they are able to support the oxidation of low-molecular intracellular compounds that possess antioxidant properties (ascorbate, NADH) and form radicals causing oxidative damage to cellular components [34]. Dopamine increases ROS generation and neuronal death [35].

It is known that the effect of dopamine varies depending on the ambient pH value. Dopamine suppresses the peroxidation of methyl linoleate in Triton X-100 detergent micelles at slightly acidic and neutral pH values. This effect does not occur at pH 8; moreover, dopamine increases peroxidation at pH ≥ 9. The prooxidant effect of dopamine and other catecholamines seems to be due to O_2 reduction with simultaneous production of a superoxide anion radical in a reaction with oxidized catecholamine. O_2 preferentially reacts with the radical anion formed in solutions with alkaline pH values, but not with the uncharged radical of catecholamine semiquinone [36]. Presumably, other phenolic biomediators have similar properties.

In our experiments, the protective effect of dopamine and norepinephrine disappears when their concentrations increase up to 3 mM (Fig. 2a). Moreover, a detrimental effect of serotonin (1–3 mM) was detected. (Fig. 2b). This may be due to manifestation of the prooxidant activity of these compounds. KCN, which increases the pH value in aqueous solutions, attenuates the antioxidant properties and increases the prooxidant properties of phenolic biomediators.

Previously, in experiments with plants, an antioxidant effect of biomediators was predominantly detected. For instance, dopamine (0.1–1 mM) in soybean roots influenced the activity of enzymes associated with ROS utilization, decreased the ROS content ($O_2^{\cdot-}$ and H_2O_2), and suppressed lipid peroxidation [37]. Our results are consistent with these observations.

CONCLUSIONS

The present work dealt with the influence of biomediators that perform neurotransmitter functions in animals on ROS formation and PCD in plants. Catecholamines (dopamine and norepinephrine) at concentrations ranging from 10 μM to 1 mM protected cells from death that was caused by KCN treatment. Serotonin, histamine, acetylcholine, and acetylthiocholine did not exhibit cytoprotective properties. Catecholamines and serotonin, unlike the other tested compounds, acted as antioxidants that reduce ROS formation in leaf epidermis (pH < 8). In general, our results complement the data on the biomediators' properties in living organisms. They demonstrate that biomediators belonging to the catecholamine group protect plant cells from cyanide-induced death. Catecholamines and serotonin prevent oxidative stress.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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