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Root exudates of wheat seedlings express antibacterial and antioxidant activity and stimulate proliferation of liver cells

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Root exudates (REs) of wheat seedlings were investigated for antioxidant, algecidic, antibacterial activity, as well as their influence on the proliferative activity of liver cells on the model of partial hepatectomy was studied. REs were obtained from 1-3-day-old wheat seedlings growing in aquatic culture. The RE of 1-3-day old wheat seedlings showed pronounced antioxidant and anti-radical activity in an *in vitro* system that was comparable to the activity of known antioxidants, such as tocopherol and free-radical scavenger - ethanol. RE of wheat seedlings had antibacterial activity against pathogens such as *Staphyloococcus aureus* and *Streptococcus pyogens*, while they stimulated the growth of nodule bacteria *Rhizobium leguminosarum*. The administration of RE in different concentrations into the microalgae culture medium of *Dunaliella viridis* showed a U-stimulatory effect on its growth, at a concentration of 0.1-1%, it was increased by 50-57% compared to the control. However, these concentrations of wheat seedlings RE did not affect the growth rate of *Chlorella vulgaris* microalgae and Spirulina platensis cyanobacteria. Intraperitoneal injection of modified RE at a dose of 0.1 mg to 1 mg / 100 g of body weight of rats after removal of 2/3 of the liver mass (partial hepatectomy) led to an increase in the rate of DNA synthesis by of 7-8 and RNA synthesis by 3-4 times compared to control.

Keywords: Antibacterial, Antioxidant activity, Green microalgae, Liver regeneration, Root exudates wheat.

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Introduction

One of the important tasks of modern biotechnology is to provide natural biologically active compounds. Plants are known to be an inexhaustible source of bioactive compounds[1-3] but along with it the extraction and cleansing is the consuming task[4,5]. All the plants are known to excrete plenty of metabolites in the environment but for roots, this ability is the most pronounced.

Plants translocate 15 to 65% of photosynthetically fixed carbon to excrete root exudates[6-8]. By excreting the root exudates, plants form symbiotic relationships with microorganisms, increase the availability of soil macro- and microelements, and protect plants from toxins, pathogens and fungi[7,9-11]. It is reported that the composition of the root exudates is defined by plant species, the phase of its development, physiological condition and complex exogenous factors of plant microenvironment (temperature, soil composition, the ratio of gases in the atmosphere, etc.)[9,12,13].

Root exudates are a complex multi-component mixture whose composition changes during the process of plant growth. It has biological activity, not only in relation to the root microenvironment but also against a wide range of plant-microbial and animal systems[14,15]. However, the practical use of the root exudates is restricted due to (i) the difficulty of obtaining exudates with defined composition, (ii) inability to control the exudates output, (iii) varying composition during excretion and storage, (iv) lack of a system to correctly assess the biological activity of exudates and (v) time-consuming methods to purify and isolate the individual components from the root exudates.

The main objective of this study was to evaluate wheat root exudates potential as biologically active components without prior separation in different test systems. For this study, *in vitro* system, bacteria, unicellular eukaryote and multicellular animal organisms were used to assess the efficacy of root exudates.

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Materials and Methods

Root exudates collection

Experiments were conducted with the root exudates of winter wheat (*Triticum aestivum* L.), cv. Donetskaya-48 seedlings. Seeds harvested in 2013 and 2014 were soaked in distilled water for 2 hours and then incubated at 24 °C in 0.005% $KMnO_4$ for 10 minutes. After which, they were again soaked in distilled water in a flat glass container at 25 °C for 21–22 hours. The germinating seeds were transferred to Petri dishes (9 cm diameter, 50 seeds per dish) and 7 mL of distilled water was added to each dish. The seedlings were grown for 1, 2 and 3 days at 24 ^oC in a phytochamber at 5 klux continuously. The root exudates collection was carried out each day, after which the seedlings were allowed to grow. The culture medium containing the root exudates was transferred from the Petri dishes to test tubes. The collected culture medium was centrifuged to pellet the starch and root border cells at 5000 g for 15 minutes at normal temperature and the supernatant was used as root exudates. Root exudates aliquots were dried at +40 ºC to a constant weight and the dried mass yield was determined for all Petri dishes of this experimental variant.

Evaluation of the antioxidant properties

Antioxidant activity of the root exudates was evaluated by their ability to reduce the accumulation of thiobarbituric acid (TBA)-active products in the suspension of yolk lipoproteins, as described by Klebanov *et al*[16].

The antiradical activity (effectiveness of the scavenging of OH• radicals) of root exudates was determined by their ability to inhibit the destruction of the deoxyribose by OH• radicals generated in the system with Fe^{2+}, EDTA, H_2O_2 and ascorbate[17].

Antioxidant and antiradical activity was expressed as a percent relative of the control. Ethyl alcohol (5 mg/mL) and tocopherol (7 mg/mL) were used as standards correspondingly[17].

Evaluation of the antibacterial activity

Antibacterial activity of root exudates of 1, 2 and 3-day old wheat seedlings was measured using the paper discs method as described by Yegorov[18]. The root exudates were sterilized by autoclaving at 121 ºC at 1 atmosphere for 20 minutes before use. *S aureus* and *S. pyogenes* were used as test organisms for determining the antibacterial activity. As a control, sterile discs soaked with sterile water were used. The antibacterial activity was determined by the area of the zone of inhibition compared to the control.

Evaluation of root exudates on *Rhizobium leguminosarum*

Effect of root exudates on *Rhizobium leguminosarum* was determined by introducing the root exudates into the bacterial medium[19] at 5% on a dry matter basis. *R. leguminosarum* growth was determined by direct microscopy, as described by Gerhardt *et al*[20]. The number of cells was determined by direct counting after 0.5, 1, 2, 3 and 4 hours after the root exudates application. The control culture grew in the medium with rhizotorphin[21].

Effect of root exudates the microalgae

The influence of root exudates on the growth and biomass yield on two microalgae *Dunaliella viridis*, *Chlorella vulgaris* and cyanobacterium *Spirulina platensis* was determined. For *D. viridisi* Artari medium[22] with root exudates at 0.001, 0.01, 0.1 1, 5, 10, and 15%, for *C. vulgaris* Tamia medium[23] and for *S. platensis* Zarucca medium[24] with 1, 3, 5, 10 and 15% of root exudates of 1-day old seedlings were prepared. *D. viridis* cells were cultured in conical flasks (V=250 mL) for 15 days, culture volume was 20 mL, and cells were counted using Goryaev camera every 2 days.

The initial concentration of cells in *D. viridis* culture was $1.3x10^6$ cells/mL, in *C. vulgaris* and *S. platensis* cultures were $3.5x10^6$ cells/mL. Cells of *C. vulgaris* were cultivated in round-bottom flasks (V=250 mL) for 30 days, and *S. platensis* in round-bottom flasks for 7 days; culture volume was 20 mL. Then algal biomass was dried and the yield of cells in the medium with different concentrations of root exudates was determined. Biomass yield of *S. platensis* and *C. vulgaris* were determined by dried weight yield and biomass yield of *D. viridis* was counted in Goryaev camera.

Determination of the DNA and RNA synthesis rate in rat liver cells after partial hepatectomy

Experiments were conducted on 3 months-old male Wistar rats. For research purpose, 25 animals were divided into 5 experimental groups with 5 animals in each group. One group was intact, and the second - control (received saline after partial hepatectomy) and 3 experimental groups: received root exudates solutions by 0.1, 0.5, and 1.0 mg of dried RE/100 g body weight one hour after partial hepatectomy respectively. The root exudates of 1-day-old seedlings were freeze-dried under vacuum. Subsequently, the

sample was dissolved in saline and administered to animals. Partial hepatectomy was performed by the classical method Higens and Anderson[25].

The rate of synthesis of DNA and RNA in the liver cells was determined 22 hours after the operation, which corresponds to the peak of their synthesis[26]. For 1 hour before animal slaughter, radioactive precursors of DNA synthesis [3] H-thymidine of 2.5 MBq/100 g body weight, and RNA - [14]C-orotic acid 1.0 MBq/100 g were administered to the animals of all groups.

Animals were euthanized by ether anaesthesia and nucleic acids were extracted from livers as described[25]. In each sample DNA and RNA, amounts were determined by the method[26]. The radioactivity of the aliquots was determined by counter Beckman (USA) and data were expressed as specific activity CPM /min•mg of DNA or RNA. The pool of radioactive labelled DNA precursors and RNA was the same in all samples, which shows the rate of synthesis of nucleic acid in the system *in vivo*.

Statistical analysis

All experiments were performed at least thrice, with each containing at least 3 repeats in each experimental point. Group comparison was performed by the Mann-Whitney test ($p < 0.05$)[27]. The statistical analyse was carried out using STATISTICA 6.0 software package.

Results

Antioxidant and antiradical activity

The redox system is one of the most important regulatory systems. Its function can be judged by the ratio of prooxidant and antioxidant activity in the body. Long-term studies of free radical oxidation have shown that the majority of known pathologies are associated with oxidative stress[29]. As is well known, drugs and biologically active compounds can be both pro- and anti-oxidant properties[30,31]. Therefore the antioxidant activity of the root exudates was carried out *in vitro*.

It was found that about 50% of the OH radicals generated in the system were scavenged in the presence of root exudates (Fig. 1a). The scavenging activity of 1- and 2-day old root exudates of wheat seedlings was more than 40% and the 3-day-old seedling root exudates showed only a slight increase (Fig. 1a). Thus, the 1 to 3-day old seedlings root exudates had pronounced ability to scavenge free radicals.

With regard to antioxidant activity *in vitro*, it was found that the root exudates had pronounced antioxidant activity (Fig. 1b) and the antioxidant activities of root exudates of 1, 2, and 3-day-old seedlings were similar. These results suggest that the root exudates also influence the redox system

Earlier, a correlation between antioxidant activity between both *in vitro* activity and *in vivo* activities of complex preparation Fungidol has been reported[32]. Thus, the *in vitro* antioxidant activity of root exudates identified herein may also be similar *in vivo*. Since the redox system is involved in the regulation of metabolism in both prokaryotic and eukaryotic cells, root exudates can be effective in a variety of organisms

Antibacterial activity

The root exudates components are known to inhibit the growth of a number of soil microorganisms[10]. It was therefore of interest to determine the effect of root exudates on the pathogenic bacteria such as *S. aureus* and *S. pyogenes*. It was found that the 1-day old seedling root exudates suppressed the growth of *S. pyogenes* for 48 hours (Fig. 2a) and the inhibition was 18-20% lower with root exudates of 3-day old seedlings compared to root exudates of 1-day-old ones.

Root exudates also inhibited the growth of *S aureus* but to a lesser extent (Fig. 2a), and the antibacterial activity of root exudates from 1st to the 3rd day was similar. These results show that the root exudates from 1 to 3-day old wheat seedlings have similar antibacterial activity against common pathogens such as the *S. aureus* and *S. pyogenes*.

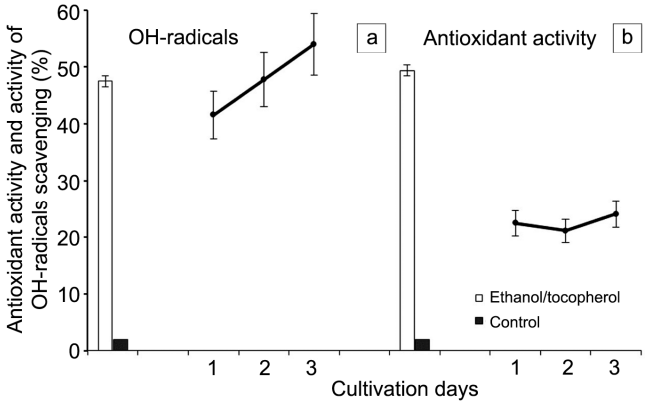

Fig. 1 — The rate of scavenging of OH-radicals and antioxidant activity of wheat root exudates of 1-, 2-, and 3-days old wheat seedlings in vitro, expressed by %. On the histogram, the corresponding activity of ethanol (5 mg/mL), α-tocopherol (7 mcg/mL) and physiological saline (lack of activity) are demonstrated.

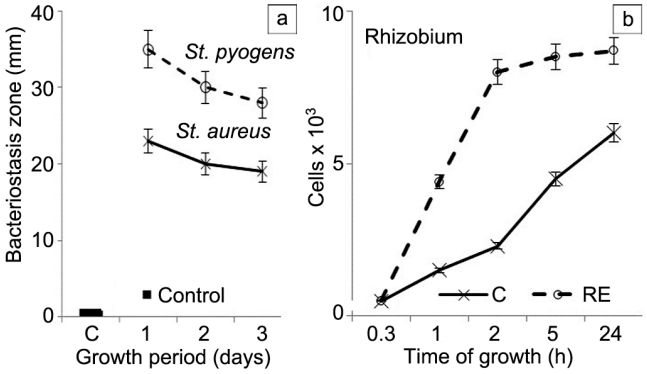

Fig. 2 — Antibacterial activity of wheat root exudates against *Staphylococcus aureus* and *Streptococcus pyogens*, growth is expressed in mm inhibition zone. The number of *Rhizobium* cells in control and in medium with root exudates at 1% of dried matter.

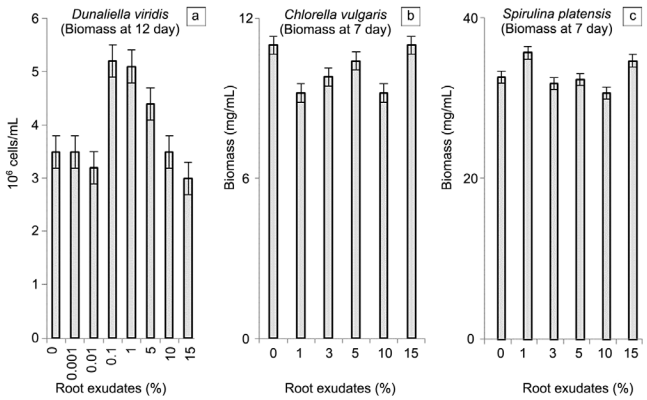

Fig. 3 — Effects of 1-day old wheat seedlings root exudates on the biomass of *Dunaliella viridis*, *Chlorella vulgaris* and *Spirulina platensis.*

The influence of root exudates on the growth of nitrogen-fixing *Rhizobium* at 1% was determined using yeast mannitol antibiotic agar medium[19] (Fig. 2b). It was found that the addition of root exudates to the growth medium decreased the length of log period and the culture reached stationary phase within 2 hours. Thus, while the root exudates inhibited the pathogenic bacteria growth, they stimulated the growth of *R. leguminosarum* suggesting the species specificity of wheat root exudates against bacteria[32].

Effects on the growth of cyanobacteria and green algae

The effects of 1-day old wheat seedlings root exudates on the growth of microalgae *D. viridis* was examined. The root exudates at 0.001-0.01% had no effect. However, at 0.1-1%, the growth was increased by 50-57% than the control (Fig. 3a). Increasing the concentration of the root exudates further up to 5, 10, and 15%, however, reduced the stimulatory effect. The effect of 3-day old wheat seedlings root exudates from 1 to 15% had no effect on biomass accumulation in green microalgae *C. vulgaris* (Fig. 3b). Similarly, the root exudates of 1-day-old wheat seedlings had no effect on the biomass yield of the cyanobacterium *S. platensis* (Fig. 3c).

Effect on rat liver regeneration after partial hepatectomy

As it is known, a partial hepatectomy induces liver regeneration through cell proliferation (increased DNA and RNA synthesis)[34]. Thus, in our study, the rate of DNA synthesis after partial hepatectomy increased 2.7 times, and RNA – 1.6 times compared to intact control levels (Fig. 4).

When the root exudates were administered to the animals at a dose of 0.1 mg/100 g of body weight one

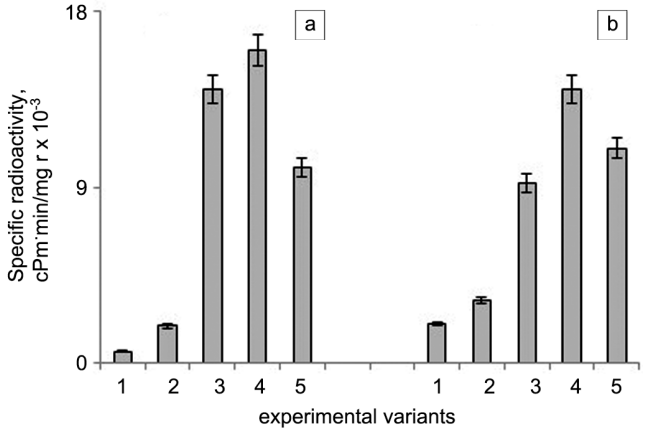

Fig. 4 — Specific radioactivity DNA (A) and RNA (B) of cell nuclei of rats. Experimental variants^ 1- intact liver; 2 – liver after 22 hours after partial hepatectomy; 3 – 5 – variant 2 + RE in doses 0.1, 0.5, and 1.0 mg/100 g body weight.

hour after partial hepatectomy surgery, and after 24 hours specific radioactivity of the DNA was determined, it was 7 times higher than the control (the partial hepatectomy without root exudates). Increasing the dose to 0.5 mg root exudates/100 g of body weight led to increase in DNA synthesis only 8 times compared with the control, and further increase in the dose to 1.0 mg/100 g body weight decreased the DNA synthesis stimulation effect, although it was yet well defined (Fig. 4). Consequently, the intraperitoneal injection of root exudates to animals caused S-shaped dose-dependent increase in the rate of DNA synthesis.

Administration of root exudates to the animals after partial hepatectomy in a dose of 0.1 mg/100 g of body weight increased the rate of RNA synthesis 3-fold compared with the control group, and when root exudates were administered at 0.5 mg/100 g of

body weight RNA synthesis increased 4.5 times as compared with the control. Large dose (1.0 mg/100 g body weight) of root exudates, as in the case of DNA has less effect.

The components of RE influence pronouncedly and significantly the rate of synthesis of DNA and RNA in the cells of the liver after partial hepatectomy, that may indicate that the RE contains substances that regulate the proliferation of not only the plant but also animal cells. It is possible that this stimulation can go for a nonspecific mechanism also.

Discussion

The experimental finding suggest the following 1) Root exudates of 1 to 3-day old wheat seedlings have strong antiradical properties, which were apparent when adding them in *in vitro* environment, 2) Root exudates inhibit the growth of pathogens, unusual for wheat seedlings *Streptococcus pyogenes* and *S. aureus* and activated growth of nodule bacteria *Rhizobium* L, capable of providing plants with nitrogen, 3) Addition of root exudates at concentrations ranging from 0.1 to 1% (V/V) in aqueous *Dunaliella* culture medium (green microalgae) increased the accumulation of biomass of and didn't affect (1-15% (V/V)) the rate of *Chlorella* (green microalga) and of *Spirulina* (cyanobacteria) growth in a wide range of concentrations, 4) Administration of root exudates to experimental rats after partial hepatectomy (2/3 of the liver was surgically removed) in doses of 0.1, 0.5, and 1.0 mg of dry matter/100 g body mass rate of DNA synthesis and RNA increased dose-dependently. It suggests a hepatotropic of root exudates and in particular the accelerating the liver regeneration process on the background of liver failure.

Addition of root exudates *in vitro* system exerted a pronounced ability to scavenge hydroxyl radicals (the common products of free radical reactions) and antioxidant activity. The natural α-tocopherol, retinol, carotenoids, and other phenolic compounds are known to pronounce antiradical activity[28,35]. The root exudates include phenolic compounds with conjugated double bonds, vitamins and other compounds exhibiting scavenger properties[21]. This explains the antiradical activity of root exudates which is already apparent in 1-day old wheat seedlings and remains unchanged from the 1st to 3rd day of growth. Consequently, the 1 to 3-day old seedlings may serve as a source of scavengers of natural origin.

These results may indicate that the root exudates components can affect the metabolism of the cells by changing the redox metabolism regulation system and at the level of second messengers. It is shown that the products of free radical reactions, along with the induction of pathological processes (in high concentrations), provide the regulation of many reactions at physiological concentrations[35]. In this regard, it is not necessary to achieve complete removal of the products of free radical reactions in biological systems, but their modulation. Probably the root exudates components can provide both *in vitro* and *in vivo*.

The root exudates contain large amounts of low molecular weight components capable to induce a series of redox reactions. These components influencing the redox potential affect one of the most ancient, fundamental, integrative levels of regulations both in pro- and eukaryotes. However, a variety of add-regulatory systems (molecular, cellular, and others) depending on species and activity of functional systems at the time of interaction will modify the signal that determines the phenotypic response in different ways (Fig. 5).

It is shown that the root exudates have a specific effect on different types of microorganisms, in the sense that they inhibit the growth of some species (*Proteobacteria, Bacteroidetes*)[36], and stimulate the other (*Bacillus amyloliquefaciens, Ralstonia solanacearum)37*.

In the present study, we revealed that *S. pyogenes* were inhibited by root exudates more than *Staphylococcus*, while they stimulate the growth of nodule bacteria of the *Rhizobium* genus. This species specificity of root exudates is known for a long time[15]. However, mechanisms of their action have not been established definitively. It is believed that inhibition of the growth of different species of bacteria is explained by evolutionary preference to certain substrates, and if for the nodule bacteria root exudates components are regulators of metabolism, the same components are foreign substances (xenobiotics) for pathogenic organisms and are able to inhibit their metabolism.

These species differences can be explained on the basis of the principle of Variable Dominating Factors (VDF)[38]. So if some kinds of components of root exudates have such an impact on hierarchical levels of formation of biological response that leads to cooperative emergent manifestations (stimulation),

and others - to the absence of any effects (neutrality), then there is the third type of response – desynchronization due to multi-directional responses on different hierarchical levels. Or, in other words, "transformation" of the response of the cell redox system is implemented in these different species. These mechanisms are little studied and it is needed to develop new experimental approaches to solve this important problem.

An important step in further research can be isolation and identification of components, depressing growth *Streptococcus* and *Staphylococcus* and investigation of their actions and the integration of molecular response may be.

In a much lesser extent, the root exudates components affect the growth rate of algae. Thus, they did not affect the growth rate of *Chlorella* and *Spirulina* in a very wide range of concentrations, while *Dunaliella* growth rate increased to 50% at a concentration of 0.1-1%, and with increasing concentrations of the root exudates components to 15%, this effect was lost and culture did not differ by the growth rate from the control level.

These differences may be associated with the fact that unlike *Dunaliella*, *Chlorella*, and *Spirulina* have no cell wall components and root exudates are able to penetrate into the cells and influence at least a redox system and thus enhance their proliferation in a certain concentration range. It is possible that in RE there are components, influencing directly on the proliferation of *Dunaliella* cells. The final solution to this problem requires special research.

The components of root exudates influence pronouncedly and significantly the rate of synthesis of DNA and RNA in the cells of the liver after partial hepatectomy, that may indicate that the root exudates contain substances that regulate the proliferation of not only the plant but also animal cells. It is possible that this stimulation can go for a nonspecific mechanism also.

It supposes that at such a pronounced dose-dependent effect of enhancing the hepatic cell proliferative activity may be caused by the influence of root exudates components on multiple levels of regulation in this process (Fig. 5). Along with these root exudates, components may affect the function of plasma membranes, thereby providing a greater degree of synchronization of the cell cycle by hepatocytes. Experimental confirmation of this is undoubted of interest.

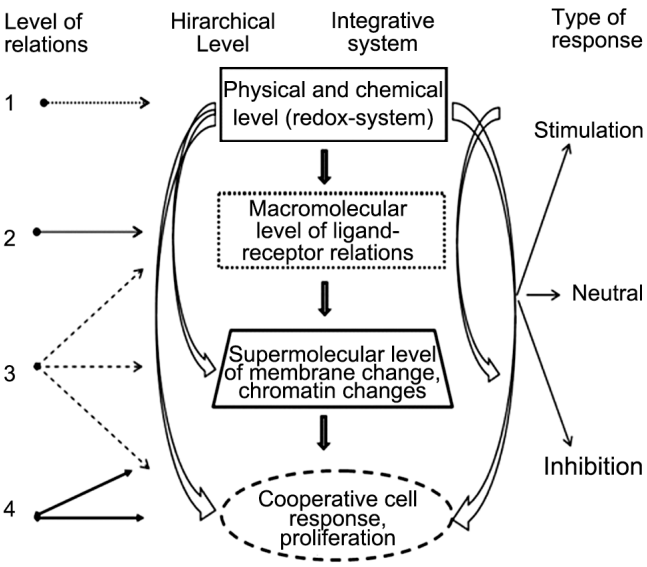

Fig. 5 — Schematic presentation of showing the hierarchical organisation of cell regulative systems. The cell is simultaneously influenced by a lot of factors. They can influence on only one or some levels. As a result, a lot of direct and indirect relations are formed and all the system answers are integrative.

In conclusion, it observed that at the root exudates have a pronounced effect on multifunctional biological systems. This effect can be explained by the principle of VDF. The essence of this principle is that the cell is acted simultaneously by many factors, however, the metabolic system currently does not react at all but only some of them, so-called dominant factors.

The dominance of a factor is temporary. Changing the conditions leads to the emergence of a new dominant factor. Changing of the dominant factors leads to variability of biological systems over time, as it is observed in the experiment.

All investigated test system in relation to the reaction to root exudates can be divided into two groups: reactive or non-reactive to the root exudates, and by response direction: stimulant and depressant; by the force response: small effects and pronounced effect (Fig. 5).

Conclusion

In conclusion, we note that the root exudates - a successful model for studying the mechanisms of interactions of polylig and multicomponent mixtures of biological origin with the organism. The root exudates may be a promising substance for pharmaceuticals and industries.

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