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Plant surface as a sensory system in allelopathic relations: 2. Cholinesterase

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ABSTRACT

Testing of cholinesterase, the enzyme that hydrolyzed acetylcholine, on the leaf surface of woody plants was done by the histochemical methods with Ellman reagent, Fast Red TR, and azo analogue of Ellman reagent dithio-bis-(p-phenyleneazo)-bis-(1-oxy-8-chloro-3,6) sodium disulfate, shortly DTPDD reagent. Last dye test was seen visually and under microscope. Inhibitors of cholinesterase neostigmine and physostigmine decreased the hydrolysis of acetylthiocholine by the cells. Presence of enzyme on plant surface shows its possible participation in allelopatic reactions between organisms containing and releasing acetylcholine.

Key Words: Acetylcholine, Ellman reagent, Fast Red TR salt, Red analogue of Ellman Reagent DTPDD, leaf surface, neostigmine, physostigmine.

Abbreviations: DTPDD - dithio-bis-(p-phenyleneazo)-bis-(1-oxy-8-chloro-3,6) sodium disulfate, Fast Red TR - Fast Red TR salt.

INTRODUCTION

The enzyme hydrolysed acetylcholine - cholinesterase at the molecular level is present in animals and plants cells (1,6-8) and microorganisms (10), since this protein encoding gene was found (16,17). Cholinesterase is used in cellular studies and as a marker on acetylcholine. The enzyme is found in the cell wall surface and some organelles in plant cell (11,12). It may be released through cell surface and often play role of the regulator of acetylcholine as signaling compond in plants. Free form of this enzyme into the environment by various organisms and proposed to neutralize the surplus of acetylcholine that affects the plant cell growth and development (11). It has been found in the models of single intact cells such as vegetative microspores of horsetail *Equisetum arvense* and pollen of knight lily *Hippeastrum hybridum* that the enzyme inhibition stimulated the germination (11).

This enzyme location in plant cells has been little studied by histochemical methods of Kelle or Karnowski-Ruth using electron microscopy (3) or using an azo dye for conventional microscopy (12). It has been shown earlier by biochemical method of Ellman that this enzyme is present in chloroplasts and by histochemical staining in the isolated nuclei and vacuoles (11,12). By vital histochemical staining methods and biochemical analysis with reagent Fast Red TR, Ellman reagent and analogue of Ellman reagent dithio-bis-(p-phenyleneazo)-bis-(1-oxy-8-chloro-3,6) sodium disulfate in intact secretory cells cholinesterase activity has been found that was concentrated mainly in plasmalemma, free space cells and cell wall (11).

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Participation of cholinesterase in allelopathic relations have been discussed by Sarma and Gupta (14) based on the experimental data with anticholinesterase substances (flavonoids, alkaloids, terpenes) and others known allelochemicals contained in weeds. The enzyme is sensitive to the compounds and this is actual new problem.

This work aimed to consider the possibilities of detecting this enzyme on the surface of leaves from allelopathically active woody species and its excretions by histochemical methods that may be useful for understanding of sensory mechanisms in allelopathy.

MATERIALS AND METHODS

1. Objects of research. The fresh leaves of following 6- spp.:

(i). *Eucalyptus cinerea* F. Muell. ex Benth. (Myrtaceae), white poplar *Populus alba* L. (Salicaceae), cherry-laurel *Prunus laurocerasus* L. Dum., Cours. (Rosaceae) and white petals mound lily *Yucca gloriosa* L. (Liliaceae), were collected from Adler culture plantations of Caucasis subtropics, Russia, on September 9-10 2019-2020 years and

(ii). common see buckthorn *Hippophae rhamnoides* L. (Elaeagnaceae) and white willow *Salix alba* L. (Salicaceae) were collected from temporary climate region of Pushchino town, Russia, on June 2022 year

2. Determination of cholinesterase activity. Reaction for the cholinesterase staining were studied on leaves, on which the drops of reagents were added on the surface. The cholinesterase activity of cells was determined by histochemical staining with reagents for cholinesterase Ellman's reagent (4), Fast Red TR salt (5), and red analogue of Ellman's reagent 2-dithio-bis-(p-phenylenazo) -bis-(1-oxy-8-chloro-3,6)-sodium disulfate, abbreviated as DTPDD reagent (12,13) before and after treatment with cholinesterase inhibitors physostigmine (eserine) and neostigmine (proserine). As a substrate, β-naphthyl acetate or acetylthiocholine was used, using the dye Fast Red TR salt (5,11) or DTPDD reagent (8,12,13), respectively. The exposure with the substrate in the medium was 40-60 min. All experiments were carried out at room temperature of 20-22 °C. The sequence of procedures was as under: The optimal substrate concentration for the hydrolytic reaction of 10^{-3} M (β-naphthyl acetate or acetylthiocholine (0.05 ml), dissolved in 0.05 M-potassium-phosphate buffer pH 7.25-7.5 was added on the sample surface. The use of this concentration has been confirmed by preliminary experiments in the range of 10^{-7}-10^{-2} M, as well as in histochemical experiments using electron microscopy (2,3). Pre-treatment (before the addition of the substrate) in treatments with cholinesterase inhibitors with proserine or eserin (10^{-6} - 10^{-4}M) lasted 20-30 min. The complete inhibition of colouration was noted at 10^{-4} M concentration, as described earlier (2,12).

3. Ozonation. Ozone for the cholinesterase inhibition (2) was produced by *Orion-Si* ozonator (*Orion* company, Russia) in special polymer camera. Short time exposure to High Concentration in total O_3 dose: 0.7 μl/L for 10 min.

4. Reagents. In the work dyes for cholinesterase Ellman's reagent, Fast Red TR salt (Sigma, USA) and 2-dithio-bis - (p-phenylenazo) - bis-(1-oxy-8-chloro-3,6)-sodium disulfate, abbreviated as DTPDD reagent and red analogue of the Ellman reagent (Chimanalyt, Russia) were used (2). Also from (Sigma, USA), cholinesterase substrates β-naphthyl acetate and acetylthiocholine, as well as cholinesterase inhibitors neostigmine and physostigmine were used.

RESULTS AND DISCUSSION

To analyze the localization of cholinesterases in intact cells and isolated organelles, histochemical staining was performed to consider the possibilities of detecting this enzyme on the surface of suitable plant species with various histochemical dyes *in situ* and in leachates (Paragraphs 1-3). To identify cholinesterase activity, experiments were conducted on the preliminary treatment of samples with cholinesterase inhibitors. Three methods were used for the histochemical analysis. The classical reagent of Ellman (4) in this reaction gave yellow colour, which, as our experience has shown as not suitable in histochemical reaction on intact tissues because the samples may have colour such as green leaves. Although yellow colour was not well marked on white petals of *Yucca gloriosa* flower too that make difficult to see the reaction with the compound. There is need to search other methods of staining. Two modes of the cholinesterase determination with Fast Red and DTPDD reagent were changed for more colouration.

1. Histochemical reactions on plant surface with Fast Red TR salt reagent.

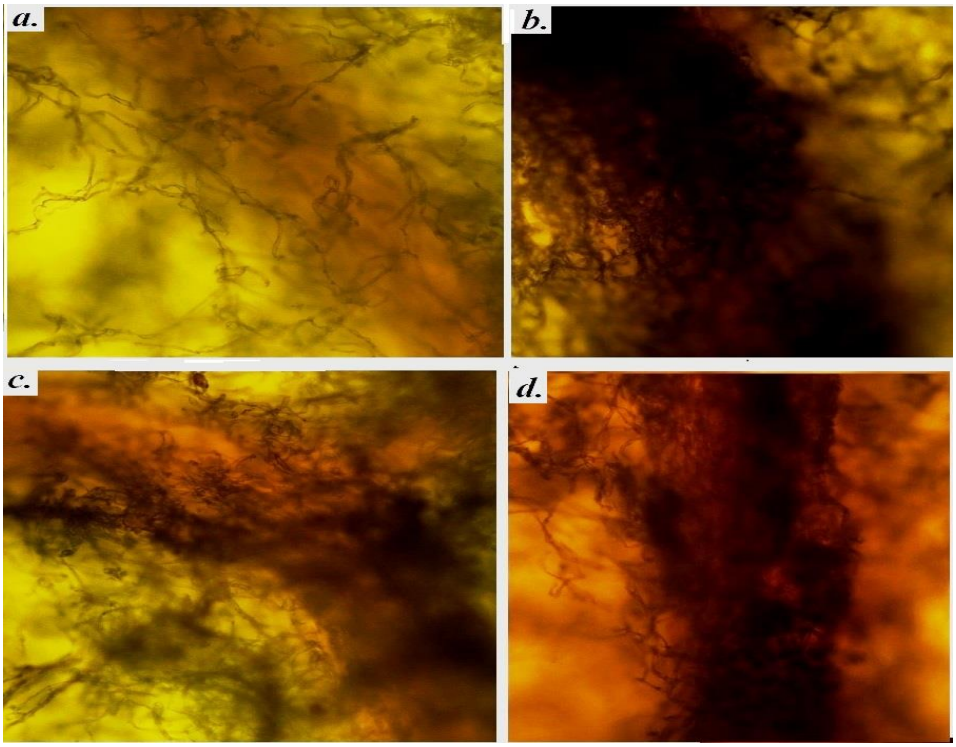

Figure 1. Histochemical staining for cholinesterase on leaf surface of *Populus alba* with Fast Red TR salt (part with the vein). a – without staining; b – control staining with β-naphthyl acetate as substrate ; c and d – treatment with neostigmine and physostigmine before histochemical reaction.

(i). *Leaf surface*: First type of reaction with Fast Red TR salt was observed with the histochemical staining with β-naphthyl acetate as the substrate (Fig.1). The greenish leaf tissue surface of *Populus alba* (*a*) after the staining reaction was coloured in red (b).This was better observed near and in the veins. Preliminary treatment with inhibitors of cholinesterases neostigmine (c) and physostigmine (*d*) decreased the colouration the control (b).

***(ii). Secretory structures on the leaf surface*:** Similar procedure with Fast Red TR salt could be used for secretory structures lying on the leaf surface. On Fig. 2 we saw the histochemical staining with Fast Red reagent in the determination of cholinesterase activity of leaf gland of *Eucalyptus cinerea*. On yellow gland surface (a) red colour was seen in transmitted light on microscope (b), which is absent in the variant with preliminary treatment of the leaf surface with neostigmine (c), inhibitor of cholinesterases in animals and plants. Similar picture was seen with other the enzyme inhibitor- physostigmine (non- illustrated) and ozone (d) that also inhibits the enzyme activity (2).

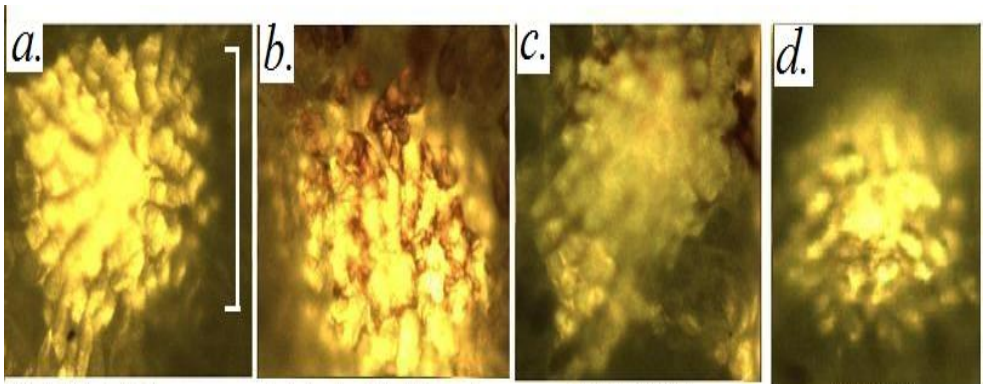

Figure 2. The histochemical staining with Fast Red TR salt reagent in testing for the cholinesterase activity of leaf gland of *Eucalyptus cinerea* . a - before histochemical staining; b - control with staining with Fast Red TR reagent; c – treatment with neostigmine 10-4 M before histochemical reaction with substrate; d – treatment with ozone 0.7 ppm 10 min before histochemical reaction with substrate. Ozone is gas that inhibits cholinesterase activity of eal (2), also decreased or diminished the colour.

2. Histochemical reactions on plant surface of petals and leaves with DTPDD reagent: We tested the histochemical method with Fast Red dye on the white petal of *Yucca gloriosa* and *Prunus laurocerasus*. However, this mehod showed weak colouration, visible only for *Yucca* sample, and it needed to use another method with red analogue of Ellmann reagent DTPDD reagent (8). For white or colour-less surface or light lower side of green leaf better result for the histochemical determination of cholinesterase was achived for *Yucca gloriosa* L. and *Prunus laurocerasus* L. (Fig. 3) in such reaction. Red colour of the dye in control with acetylthiocholine as substrate converted to bluish (1). If the samples were preliminary treated with neostigmine (2) and physostigmine (3) before the addition of the substrate, we saw reddish or colour

less drops on the surfaces. The hydrolysis of acetylthiocholine as the substrate has been inhibited by neostigmine and physostigmine. This confirms the presence of cholinesterase on the leaf surfaces of the plants.

Figure 3. Histochemical staining for cholinesterase of the flower petal from *Yucca gloriosa* (upper side) and leaf surface of *Prunus laurocerasus* (lower side) with DTPDD reagent with substrate acetylthiocholine. 1 – without inhibitors; 2 and 3 – treatment with neostigmine and physostigmine, relatively, before– treatment with neostigmine 10^{-4} M before histochemical reaction with substrate

3. Cholinesterase reaction of leaf leachates: We also tried to use the 10 min water leachates from the leaves of *Salix alba* and *Hippophae rhamnoides* to determine the enzyme activity with DTPDD reagent (Table 1). In both variants, we saw the hydrolysis of acetylthiocholine as the substrate, which has been inhibited by neostigmine and physostigmine.

Sensory systems of leaf surface are considered as the acceptor of external signals-allelochemicals. In our case, there are compounds in excretions of living organisms in biocenosis from microbiota, plants and animals. In the systems, there are compounds, which

participate in the recognition of the allelochemicals and react with them. In many cases, nature of possible sensors of plant-acceptor of allelochemicals on the surface is unknown yet. First direction includes events occurred on the leaf surface (stomata, free parts and secretory structures) as a whole, when seen with microscope. The surface also may be covered by excretions of own cells, including enzymes and secondary metabolites as well as stationary components of wax, cuticle and cell walls. To our regret, there are no special microscopic research of cell surface in a connection with allelopathy.

Exogenous acetylcholine from the excretions of plants and animals serves as a signal and growth regulator (9,11). It may stimulate the seedling growth, the fertilization and formations of fruits (8). The leaf surface may be observed as a sensory system, including cholinesterases as the enzyme-sensor, which regulates the interactions with external compounds, both plant - excreted allelochemicals and secretions from insects (1,7,15) and microorganisms which contain acetylcholine (10).

Table 1. The determination of cholinesterase activity with DTPDD reagent. Leachates by water were 1: 10 (weight/ volume).

Plant species	Absorbance at 590-600 nm		
	Control	+ neostigmine	+ physostigmine
Hippophae rhamnoides	0.125±0.010	0.048±0.005	0.028±0.004
Salix alba	0.064±0.02	0.026±0.015	0.014±0.01

Weeds contain allelochemicals-inhibitors of cholinesterases, which possibly are also deterrents against herbivory growth in the same habitat. Sharma and Gupta (14) did experiments and summarized information for substances with an anticholinesterase activity in weeds. Leaves and roots/tubers of the weeds were tested for presence of anticholinesterase agents. Most weeds tested inhibited acetylcholinesterases from both animal (Eel) and plants (tomato and wheat). From ethanolic extract of roots of 45 weeds analyzed, 12 showed 100 % and 27 showed 50 % inhibition of the enzyme from animal source while methanolic extract of leaves of 30 weeds showed 50 % inhibition and 17 weeds showed 100 % inhibition of enzyme from the same source. Anticholinesterases from roots of 14 weeds showed 50 % inhibition of cholinesterases from wheat and weeds like *Bidens biternata*, *Coronopus didymus*, *Prosopis juliflora*, *Ricinus communis*, *Verbascum chinense*, *Veronica agrestis* completely inhibited the enzyme. Leaf extract of 75 % of these weeds inhibited the wheat enzyme by more than 50 % and 15 weeds caused complete inhibition of the enzyme. Root extract of the weeds tested showed more than 50 % inhibition of acetylcholinesterase from tomato leaves. The excretions of *Cyperus rotundus* inhibited the enzymes from animal and plant sources and also inhibited germination and seedling growth in wheat and tomato. One could see main groups of inhibitors of plant and animal cholinesterases, mainly alkaloids harmaline, berberine and sanguinarine, as well as phenols scopoletin, naringenin and aromatic compounds like cineole (14).

CONCLUSIONS

Enzyme cholinesterase activity was tested on the leaf surfaces of 5-studied plants *Eucalyptus cinerea* F. Muell. ex Benth. (Myrtaceae), white poplar *Populus alba* L. (Salicaceae), cherry-laurel *Prunus laurocerasus* L. Dum., Cours. (Rosaceae) , white petals

mound lily *Yucca gloriosa* L. (Liliaceae) and in leachates from leaves of common see buckthorn *Hippophae rhamnoides* L. (Elaeagnaceae) and white willow *Salix alba* L. (Salicaceae) by histochemical and biochemical reactions with some reagents. The enzymes are component of sensory systems accepting acetylcholine derived from different organisms participating in chemical interrelations: from plant-plant, animal-plant or microorganism-plants.The surface location of the enzyme permits to regulate amount of acetylcholine, which affects the growth of seedlings, spores and pollens. This is new line in allelopathic studies related to contacts between organisms in biocenosisis

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CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct.

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PUBLISHER NOTE