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ORIGINAL ARTICLE

THE IMPORTANCE OF OXIDATIVE PHOSPHORYLATION UNCOUPLING SYSTEMS FUNCTION FOR SURVIVING OF WINTER WHEAT SEEDLING SHOOTS DURING SHORT-TERM COLD STRESS

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Abstract— The influence of uncoupling of oxidative phosphorylation in mitochondria on surviving of winter wheat seedling during low-temperature stress was investigated. It was shown that infiltration of winter wheat seedling by exogenous uncouplers caused the increase of oxygen consumption, seedling's temperature and surviving. Vice verse inhibition of electron transport through the main cytochrome and alternative pathways decreased surviving of winter wheat seedlings during cold stress..

Key words: Triticum aestivum L. / uncoupling of oxidative phosphorylation / alternative oxidase / thermogenesis / surviving

INTRODUCTION

It is known that living cell generate heat during all metabolic processes, especially during ATP hydrolysis and mitochondrial respiration because of partial uncoupling of oxidative phosphorylation (Skulachev, 1999). This partial uncoupling in plants depends on different biochemical mechanisms function – dissipation of $\Delta\bar{\mu}_{H^+}$ because of function of ADP/ATP antiporter and UCP-like protein by "fatty acid cycling" mechanism (Skulachev, 1999) and function of other specific electron transport pathways as alternative oxidase (AOX) in practically all plant species (Siedow and Umbach, 2000) and uncoupling protein CSP 310 in cereals (Kolesnichenko et al., 2002). Participation of each mentioned above mechanism in heat generation was shown previously, especially for AOX during flowering of *Aroidae* (Wilson and Smith, 1971; Meeuse, 1975), when heat generation is so strong that the difference between temperatures of plant tissue and surrounding air amount to 10 and even 20 ^{0}C that on 35 ^{0}C higher than temperature of the environment (Nagy et al., 1972; Knutson, 1974). There are some data shows that in so-called "non-thermogenic" plants at certain condition thermogenesis occur too (Moynihan et al., 1995; Vojnikov et al., 1984). Thus, thermogenesis in plant tissues was detected during short-term cold stress, when in non-hardened cold-resistant plants heat generation during first 20 – 30 min of cold shock was detected (Vojnikov et al., 1984; Grabelnych et al., 2003).

At the same time the role of thermogenesis during cold stress is not exactly determine yet and under consideration now (Moynihan et al., 1995; Breidenbach et al., 1997; Jarmuszkiewicz et al., 2001). Some authors consider that in plants under such conditions AOX participate in thermoregulation, which allows plants to survive during stress conditions (Ordentlich et al., 1991; Nevo et al, 1992; Moynihan et al., 1995). At the same time other researchers consider that the increase of plant respiration during cold stress because of activating of is not enough for significant increase of tissue temperature and therefore did not influence on plant surviving during cold stress (Breidenbach et al., 1997; Jarmuszkiewicz et al., 2001).

On the other hand, AOX is not the unique thermogenic system in plants. Recently other thermogenic systems such as Plant Uncoupling Mitochondrial Protein (PUMP) (Vercesi et al., 1995; Laloi et al., 1997) and stress uncoupling protein CSP 310 (Kolesnichenko et al., 1996) were found in plants. If AOX and PUMP are inner mitochondrial membrane proteins, CSP 310 is a cytoplasmic protein and can reversibly associate with outer mitochondrial membrane during low-temperature stress (Kolesnichenko et al. 2000, 2005). It was found that CSP 310 differs by its mechanism from these proteins (Kolesnichenko et al., 2002) but all of them participate in heat generation in winter wheat seedling shoots during cold shock (Grabelnych et al., 2003). Further it was shown that CSP 310 shunts electrons around the main cytochrome pathway of the mitochondrial respiratory chain, i.e. electron flow

bypasses ubiquinone and complex III via CSP 310 (Kolesnichenko et al., 2005). The fact that CSP 310 can rapidly associate and dissociate with mitochondrial outer membrane and its ability reduce oxidized form of cytochrome c in vitro and in organello shows that this protein can be localized in points of tightly contact of outer and inner mitochondrial membranes where it can transfer electrons to cytochrome c and further to complex IV (Kolesnichenko et al., 2005).

At the same time thermogenesis is not the only function of such systems in mitochondria. Other important function of such mitochondrial systems is to reduce reactive oxygen species formation under cold stress (Jarmuszkiewicz et al., 2001; Kolesnichenko et al., 2001). Previously it was found that activation of plant uncoupling mitochondrial systems decrease the rate of lipid peroxidation during cold shock (Kolesnichenko et al., 2001).

So, the aim of this work was to compare the influence of uncoupling of oxidative phosphorylation by natural and artificial uncouplers on oxygen consumption and plant surviving during cold shock and to study the influence of plant uncoupling mitochondrial systems inhibitors on oxygen consumption, lipid peroxidation and surviving of winter wheat seedling shoots during cold shock.

MATERIALS AND METHODS

Shoots of 3-day-old etiolated seedlings of winter wheat (*Triticum aestivum* L., winterhardy cv. "Irkutskaya ozimaya") were used in the study. Seedlings were grown on wet filter paper in thermostat at 26 ^{0}C.

Winter wheat seedlings were chilled with the temperatures ranging from 20 to 0, -4 ^{0}C for 1 h (cold shock). In the study of an influence of inhibitors on surviving, lipid peroxidation, thermogenesis and oxygen uptake at winter wheat seedlings 3 g of shoots were infiltrated by 1) procaine (40 mM) (Scherphor et al., 1972), 2) KCN (10 mM) (Vojnikov et al., 1984), 3) BHAM (25 mM), 4) BHAM (25 mM) + KCN (10 mM), 5) CSP 310 (1 mg/ml) and 6) CCCP (1 – 10 μM) for 40 min. After infiltration, the shoots were washed and excess moisture was removed from the shoot surface with filter paper. The temperature of chilled seedlings was recorded by a copper-constantan thermocouple (wire diameter 0.1 mm) connected to the input of a high-sensitive microvoltmeter (Vojnikov et al., 1984). The sensitivity of this thermocouple was 0.025 ^{0}C. For the measurement, seedlings shoots (3 g) were tightly packed in a small container at 20 ^{0}C and then transferred to thermostat with experimental temperature (-4 ^{0}C). Temperature changes were recorded in the container filled with shoots or in plant shoots for 1 h. The shoots sample then was placed in hot water (95 ^{0}C) to stop all metabolic processes. Excess moisture was removed from the shoot surface with filter paper and the temperature changes were recorded in samples cooled from 20 ^{0}C to experimental temperature. Thus, we obtained temperature curves following chilling with one tissue sample for living and for dead tissue and calculated the temperature difference (ΔT^0) between "killed" and "alive" seedlings shoot tissue.

The oxygen uptake of winter wheat shoots was recorded polarographically at 27 ^{0}C using a platinum electrode of a closed type in 1.4 ml volume cell (Estabrook, 1967). The reaction mixture contained 50 mM KH_2PO_4 pH (5.2), 50 mM sucrose. Polarogramms were used to calculate the oxygen uptake of shoot tissue.

The rate of lipid peroxidation was determined by measuring of the primary products of lipid peroxidation – conjugated diene formation. For preparation to measurement the dienic conjugate contents winter wheat shoots were pound in mortar with Tris – HCl buffer (pH 7.8) and lipids were extracted by hexane – isopropanol (1:1 v/v) mixture (9 ml per 1 ml of the sample) by shaking. After shaking 1 ml H_2O was added to the mixture to stratify hexane and isopropanol phases. Measurement of dienic conjugate contents was made in hexane phase at 233 nm on spectrophotometer "SF-46" ("LOMO", USSR). The dienic conjugate contents in the sample were calculated according to 233 nm molar extinction coefficient to polyunsaturated fatty acids conjugated dienes $2{,}2 \times 10^5 \times M^{-1}$ sm $^{-1}$ (Recknagel and Ghoshal, 1996).

To measure the surviving of winter wheat shoots after cold shock the seedlings were infiltrated by H_2O, CSP 310 or CCCP for 40 min and were subjected to cold shock in thermostat at –4 ^{0}C for 20 min. After the temperature treatment the seedlings were replaced into dish on wet filter paper and were grown at 26 ^{0}C for 3 days. The number of living seedlings was determined and the percentage of the survived seedlings was calculated.

CSP 310 and anti-CSP 310 antiserum were obtained using the method described previously (Kolesnichenko et al., 1996). It was previously shown that infiltration by CSP 310 caused the increase of CSP 310 subunits content in CSP 310-infiltrated shoots and the increase of their oxygen consumption, moreover, infiltration of winter wheat shoots by anti-CSP-310 antiserum caused the decrease of CSP 310 subunits content shoots and the decrease of their oxygen consumption unlike the non-immune antiserum infiltration (Kolesnichenko et al., 2003). Based on these data we used the same infiltration method in our experiments.

All experiments were made in ten separate preparations. The data obtained were analysed statistically, i.e. arithmetic means and standard errors were determined.

RESULTS AND DISCUSSION

To determine physiological role of oxidation phosphorylation uncoupling during low temperature stress we use two uncoupling agents: natural uncoupler - stress uncoupling protein CSP 310 and artificial uncoupler CCCP with well-studied influence on mitochondrial oxidative phosphorylation and compare their influence on oxygen uptake,

temperature and surviving of winter wheat shoots during cold shock. Because we did not found the data about the influence of CCCP concentration in incubation medium on winter wheat seedling shoots oxygen consumption in the literature, firstly we studied the effect of CCCP concentration on them. It was found that the optimal concentration that caused the most pronounced increase (30%) of seedling shoots oxygen consumption is 2 μM (Fig. 1). Further increase of CCCP concentration did not caused such pronounced increase and concentrations higher than 5 μM even inhibited oxygen consumption of seedling shoots apparently because of its toxic effect. Therefore in all experiments we used CCCP solution with concentration 2 μM for infiltration of shoots.

In spite of the fact that CCCP is well known as an uncoupling agent, its influence is well studied only *in vitro* and *in organello* but not on the level of the whole plant organism. It is not established yet, whether the increase of oxygen consumption after plant infiltration by this uncoupler is the consequence of it's uncoupling of oxidative phosphorylation or the consequence of damage of cellular organelles or biochemical mechanisms. So, it was necessary to determine, if CCCP infiltration-dependent increase of oxygen consumption is reversible and have infiltration of CCCP any influence on seedling shoots surviving after this treatment or not. So we study the aftereffect of CCCP infiltration on winter wheat seedling shoots. The data obtained showed that at the first day after infiltration seedling shoots oxygen consumption was about 30% higher than in control, but henceforth this difference disappeared and after five days there we did not detect any difference between these variants (Fig. 2). It is necessary to note that both control and CCCP-treated seedling shoots have 100% surviving after 10 days of grow. Therefore we can conclude that CCCP dependent uncoupling in winter wheat seedling shoots is reversible, did not depend on plant damaging and did not influence on their surviving in control conditions.

The next step of the study was to compare the uncoupling influence of CCCP and CSP 310. It was found that infiltration of seedlings by 1 mg/ml (3.2 nM) water solution of CSP 310 caused about 20% increase of oxygen consumption (Table 1). It was about 2/3 of CCCP effect on oxygen consumption.

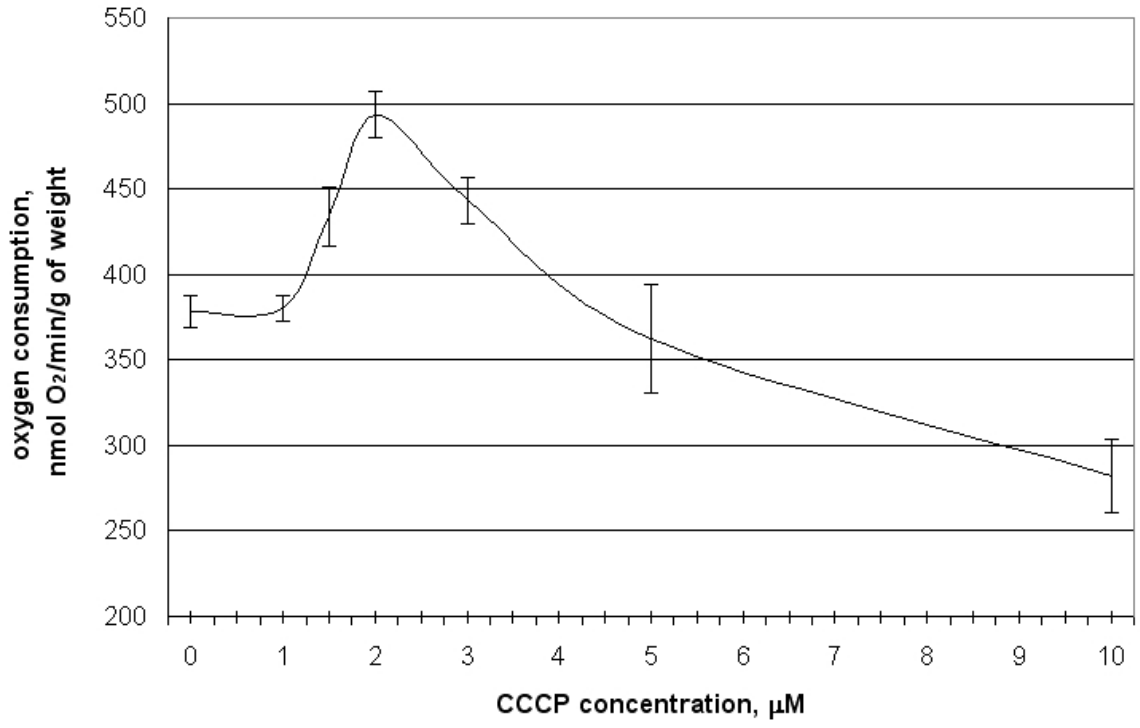

Fig. 1. The influence of CCCP concentration in infiltration medium on the oxygen consumption of winter wheat seedlings shoots. M±SD, n=3

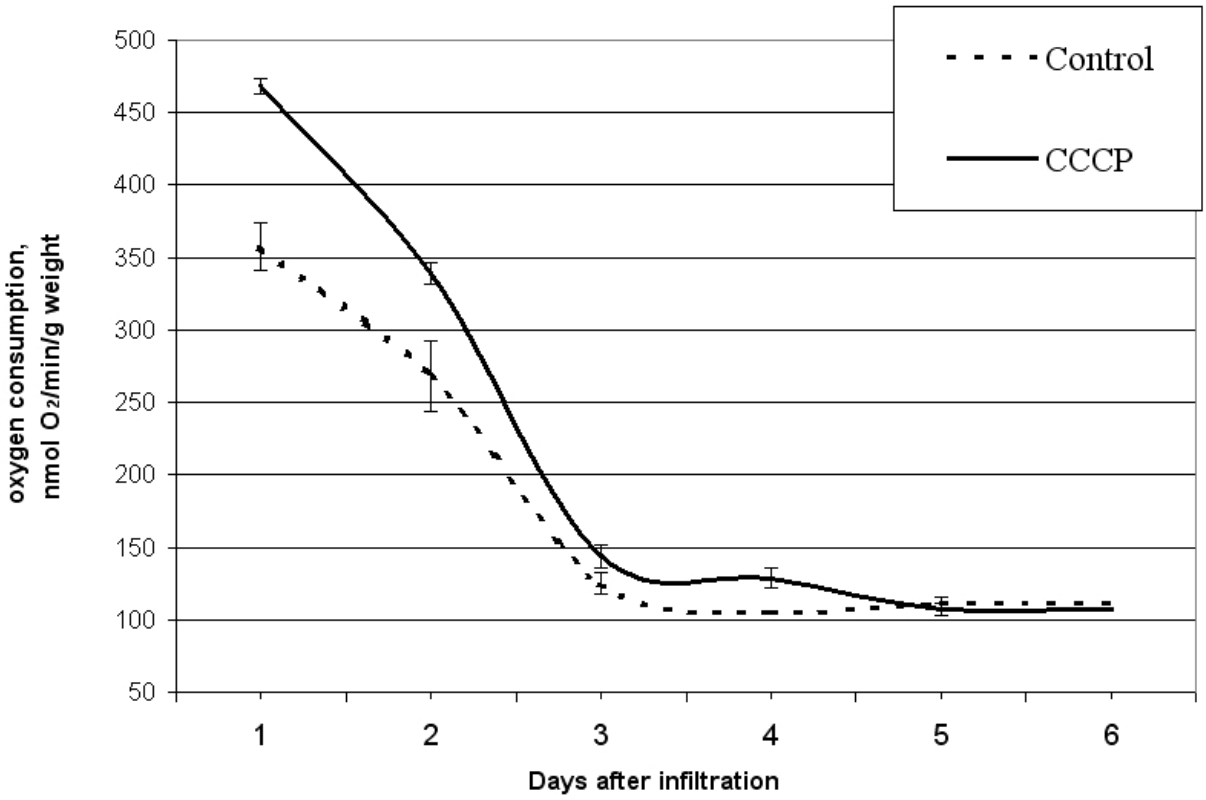

Fig. 2. The dynamic of winter wheat seedlings shoots oxygen consumption after infiltration of water ("Control") and 2 μM CCCP ("CCCP"). M±SD, n=3.

Table 1. The influence of infiltration of water ("control"), CCCP ("CCCP") and CSP 310 ("CSP 310") on oxygen consumption of winter wheat seedlings shoots. M±SD, n≥6

Variants	Oxygen consumption, nmol O_2/min/g of weight
Control	362,44±12,92
CCCP (2 μM)	473,19±11,16
CSP 310 (1 mg/ml)	439,50±12,93

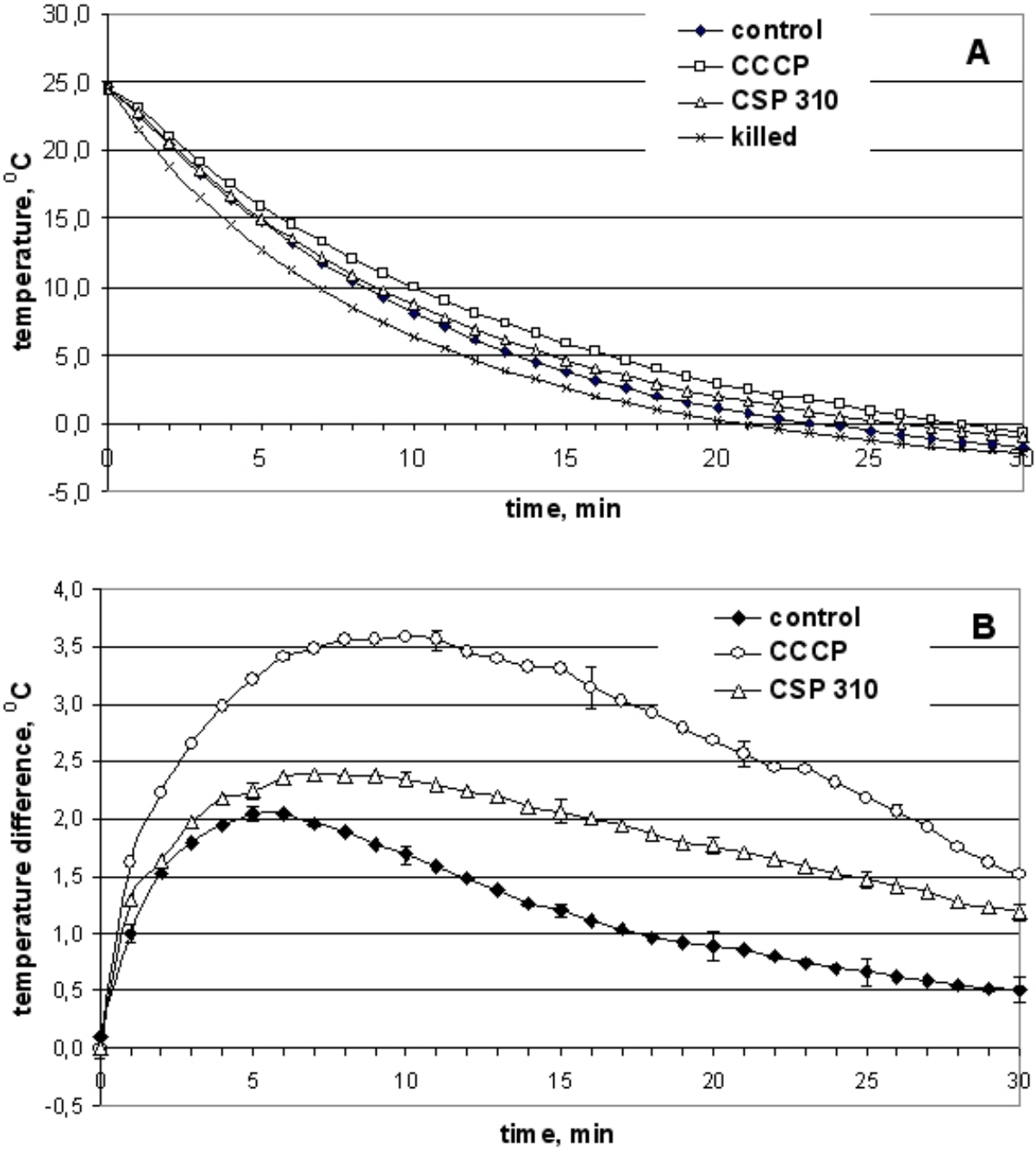

Fig. 3. The temperature of "control" (water-infiltrated), CCCP-infiltrated, CSP-310 infiltrated and "killed" winter wheat shoots during cold shock at –4 ^{0}C (A) and the temperature difference between "living" and "killed" control, "living" and "killed" CSP 310-infiltrated and "living" and "killed" CCCP-infiltrated winter wheat shoots during cold shock at –4 ^{0}C (B). M$\pm$SD, *n*=6.

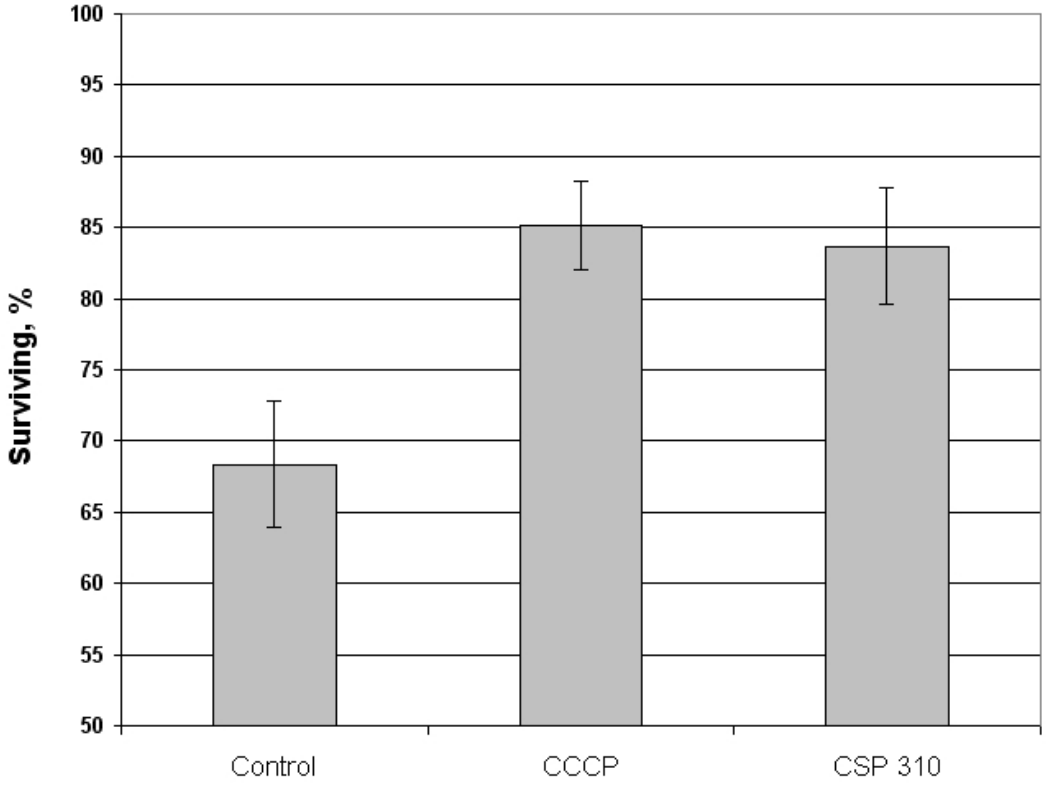

Fig. 4. The influence of infiltration by water ("Control"), 2 μM CCCP ("CCCP") and 1 mg/ml CSP 310 ("CSP 310") on surviving of winter wheat shoots after short-term cold stress (-4 ^{0}C, 20 min). M±SE, n≥10

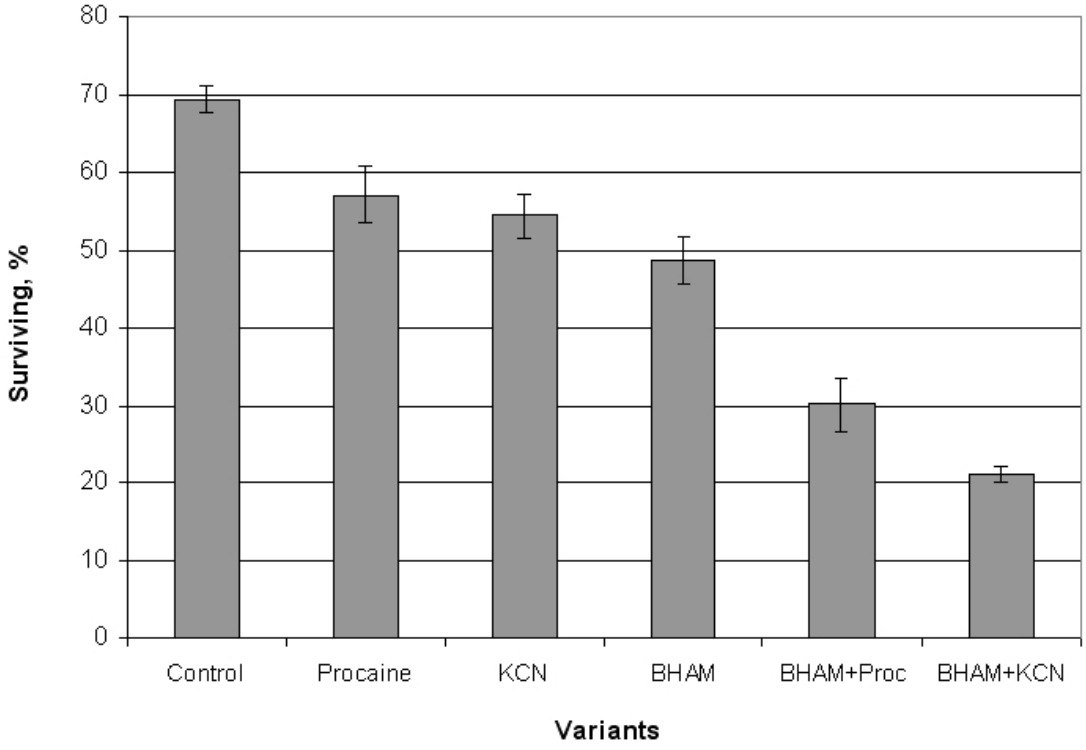

Fig. 5. The influence of infiltration by water ("Control"), 40 mM procaine ("procaine"), 10 mM KCN ("KCN"), 25 mM BHAM ("BHAM"), mixture of 25 mM BHAM and 40 mM procaine ("BHAM+Proc") and mixture of 25 mM BHAM and 10 mM KCN ("BHAM+KCN") on surviving of winter wheat shoots after short-term cold stress (-4 ^{0}C, 20 min). M±SE, n≥10

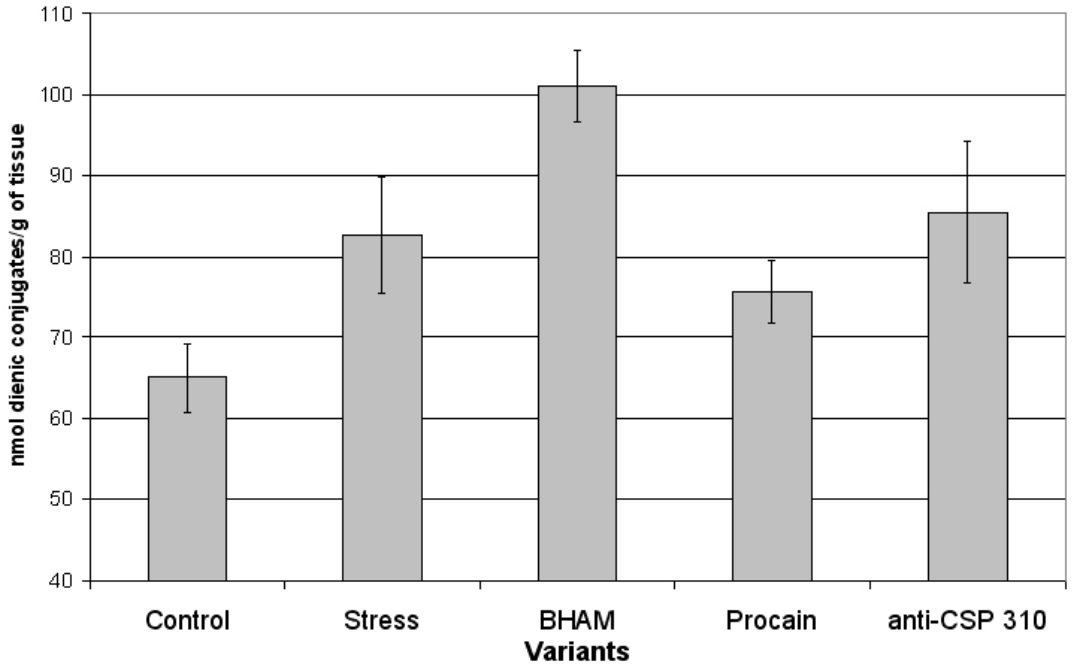

Fig. 6. The influence of known plant uncoupling mitochondrial systems inhibitors on lipid peroxidation in winter wheat shoots during cold stress (-4 ^{0}C, 20 min). M±SE, n=4.

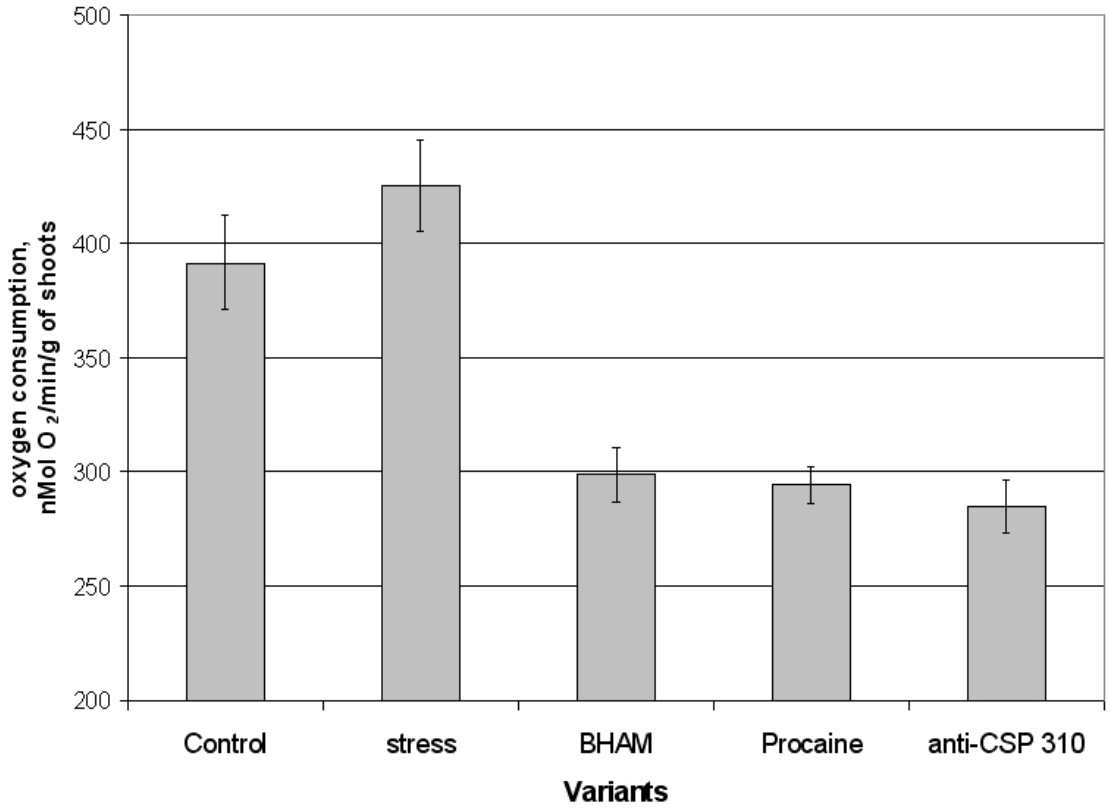

Fig. 7. The influence of known plant uncoupling mitochondrial systems inhibitors on oxygen consumption of winter wheat shoots during cold stress (-4 ^{0}C, 20 min). M±SE, n=4.

These treatments also caused the increase in winter wheat shoots temperature during cold shock (Fig. 3). The temperature difference between "CCCP-infiltrated" and "killed" and "CSP 310-infiltrated" and "killed" as compared with "water-infiltrated" and "killed" shoots is increased during the first 10-15 min of cold shock and amount to 3.5 ^{0}C and 2.5 ^{0}C (Fig. 3). Comparing the data about CCCP- and CSP 310-

dependent increase of oxygen consumption (Table 1) with data about the influence of CCCP and CSP 310 infiltration on the temperature of winter wheat seedling shoots during cold shock (Fig. 3) we can conclude that they were similar: CSP 310 caused 50% increase of shoots' temperature and CCCP caused 75% increase of their temperature during cold stress as compared to control shoots, that is about 2/3 ratio too. So we can conclude that the rate of thermogenesis caused by uncoupling agents depends on their influence on oxygen consumption.

The study of the influence of uncoupling on surviving of winter wheat seedling shoots during cold shock showed that both infiltration by CCCP and infiltration by CSP 310 caused the increase of seedling surviving during cold shock (Fig. 4), but in this case we did not detect difference between these treatments. Both of them caused about 25% increase of surviving after 20 min cold shock at $-4\ ^0C$.

It was previously shown that infiltration of winter wheat seedling shoots by some inhibitors of mitochondrial electron transport pathways caused the decrease of seedling shoots temperature during cold shock (Grabelnych et al., 2003). In the next set of experiment we try to determine how these treatments influence on seedling shoots surviving during cold shock ($-4\ ^0C$, 20 min). It was found that infiltration by KCN that blocks terminal oxidase caused only about 20% decrease of seedling shoots surviving (Fig. 5) in spite of the fact that this infiltration significantly decrease seedling shoots oxygen consumption and temperature (Grabelnych et al., 2003). Infiltration of seedling shoots by BHAM caused 30% decrease of their surviving that shows importance of AOX function for winter wheat during cold stress. Infiltration by procaine, that inhibits phospholipase A_2 activity (Scherphor et al., 1972) and decrease free fatty acid content in seedling shoots (Vojnikov et al., 1983) caused the decrease of seedlings shoots surviving during cold shock too (Fig. 5) that shows the participation of PUMP, which function depends on free fatty acids content (Jezek, 1999), in plant protection against low-temperature stress.

The study of influence of mixtures of inhibitors on seedling shoots surviving during low-temperature stress showed that both mixtures used (BHAM+KCN, which should inhibits all known mitochondrial uncoupling systems, and BHAM+procaine, which should keep function only CSP 310) inhibited plant surviving more than each inhibitor individually (Fig. 5). It is necessary to note that infiltration by mixture BHAM+KCN exert more influence on plant surviving than BHAM+procaine, which shows the importance of plant stress protein CSP 310 for plant surviving during cold shock.

So, inhibition of each mitochondrial system studied caused the decrease of plant surviving during short-term low temperature stress. The data obtained are well correlated with previously published data about the influence of inhibition of these mitochondrial uncoupling systems on the temperature of winter wheat seedling shoots during cold shock (Grabelnych et al., 2003).

Now most of researchers consider that the main function of plant uncoupling proteins is to protect plants against oxidative stress that is concomitant to cold stress. Indeed, previously we report that infiltration of winter wheat seedling shoots by activators of plant uncoupling mitochondrial systems - by pyruvate, which activate AOX (Vanlerberghe et al., 1999), linoleic acid, which activate PUMP (Jezek, 1999) and CSP 310 (Voinikov et al., 1998) decrease the formation of dienic conjugates during cold shock (Kolesnichenko et al., 2001). So, the aim of following part of the study was to determine, is inhibition of mitochondrial uncoupling systems cause the increase of dienic conjugates formation during cold shock or not.

The data obtained showed that, if infiltration of seedling shoots by BHAM caused the increase of dienic conjugates formation during cold shock as it was expected according to this hypothesis, infiltration of seedling shoots by other inhibitors used in this experiment did not influence on dienic conjugates formation (Fig. 5), although these treatments decreased tissue oxygen consumption (Fig. 7) and plant surviving during cold stress (Fig. 5). Therefore, we can suppose, that this fact is due to the complexity of plant defense system against oxidative stress. Inhibition of some of antioxidant systems should activate other antioxidant systems, which should protect plants against oxidative stress. On the other hand, based on these data, we can conclude that protection against oxidative damage during cold stress is not the only function of plant mitochondrial uncoupling systems during cold stress.

So, we can conclude that infiltration of winter wheat seedling shoots by oxidative phosphorylation uncouplers increase of their oxygen consumption, temperature and surviving during short-term cold stress. Inhibition of known plant mitochondrial uncoupling systems decrease the tissue oxygen consumption, temperature and surviving of winter wheat seedling shoots during short-term cold stress, which shows the physiological importance of these systems function for plant protection against cold stress.

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