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EXPERIMENTAL ARTICLES

Respiratory Activity and Naphthoquinone Synthesis in the Fungus *Fusarium decemcellulare* Exposed to Oxidative Stress

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Abstract—The effect of oxidants (hydrogen peroxide and juglone) on the growth, respiration, and naphthoquinone synthesis in the fungus *Fusarium decemcellulare* was studied. The addition of the oxidants to the exponential-phase fungus inhibited cell respiration (either partially or completely, depending on the oxidant concentration), culture growth, and naphthoquinone synthesis. The treatment of fungal cells with nonlethal concentrations of H_2O_2 (below 0.25 mM) and juglone (below 0.1 mM) induced the resistance of cell respiration to cyanide. The residual respiration in the presence of cyanide could be inhibited by benzohydroxamic acid, indicating the occurrence of alternative oxidase. Increased concentrations of oxidants (0.25 mM juglone and 0.5 mM H_2O_2) rapidly and irreversibly inhibited cell respiration. These observations suggest that the mitochondrial respiratory chain of fungal cells exposed to oxidative stress is subject to the action of active oxygen species. The treatment of fungal cells with nonlethal concentrations of H_2O_2 and juglone activated cellular glutathione reductase and glucose-6-phosphate dehydrogenase, which are protective enzymes against oxidative stress.

Key words: fungus, oxidative stress, hydrogen peroxide, juglone, adaptation, glutathione reductase, glucose-6-phosphate dehydrogenase, alternative oxidase, naphthoquinones.

Under aerobic conditions, many microorganisms suffer from the action of active oxygen species (AOS), including hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), and hydroxyl ions (OH^-). These forms of active oxygen can rapidly kill cells because of their ability to induce lesions in DNA and RNA and to oxidize proteins and lipids, thus inflicting damage on various cellular structures [1, 2].

It is known that one of the primary responses of plants to phytopathogenic invasion is the activation of O_2^- and H_2O_2-generating processes [3–7]. Many plants are also able to produce defense autoxidizable quinone compounds, which oxidize NAD(P)H in plant and phytopathogenic cells with the formation of superoxide radicals and hydrogen peroxide [8]. In response, the phytopathogenic fungi activate their synthesis of naphthoquinones, which possess phytotoxic action and can suppress the defense reactions of plants [9].

In the previous work [10], the treatment of the fungus *Fusarium decemcellulare* with nonlethal concentrations of hydrogen peroxide and juglone was found to induce the resistance of fungal cells to high concentrations of these oxidants and to enhance the catalase and superoxide dismutase (SOD) activities of the cells.

The aim of the present work was to study the adaptation of *F. decemcellulare* cells to oxidative stress, with particular emphasis on the effect of hydrogen peroxide and juglone on cell respiration, naphthoquinone synthesis, and the glutathione reductase and glucose-6-phosphate dehydrogenase activities of the cells.

MATERIALS AND METHODS

The phytopathogenic fungus *Fusarium decemcellulare* F-1179 used in this study was obtained from the All-Russia Collection of Microorganisms (VKM). The fungus was cultivated at 29°C on a shaker (200 rpm) in 700-ml flasks with 100 ml of Reader medium [11] containing 2% glucose as the source of carbon and energy. The medium was inoculated with a one-day mycelium. Growth was monitored by determining the dry weight of the fungal biomass.

The total amount of naphthoquinone pigments in the culture liquid was evaluated spectrophotometrically at 500 nm using the coefficient of millimolar extinction equal to 7.3 [12]. Cells from the culture liquid were preliminarily removed by centrifugation.

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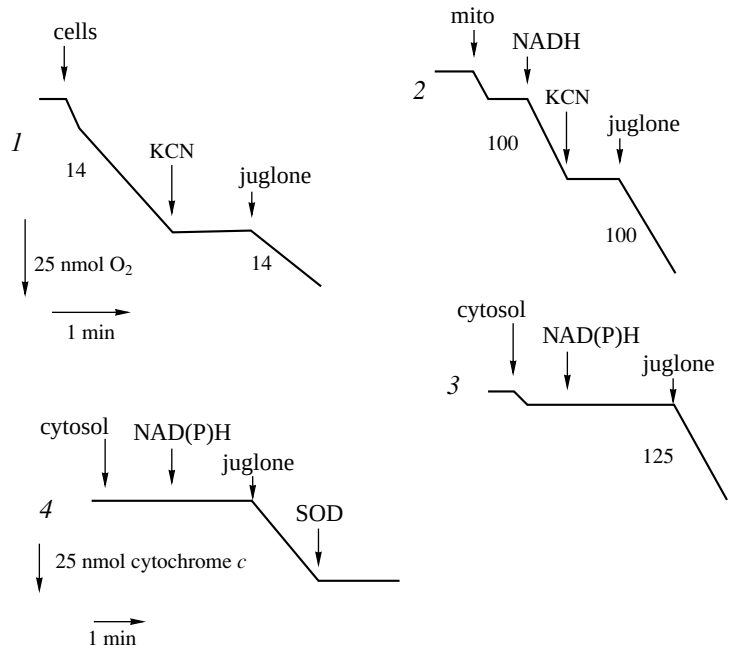

Fig. 1. The effect of juglone on the consumption of oxygen by (*1*) *F. decemcellulare* cells, (*2*) mitochondria (mito in short), and (*3*) cytosol. Curve *4* illustrates the reduction of cytochrome *c* due to the formation of superoxide radicals by the cytosolic fraction of cells in the presence of NAD(P)H. Cells, mitochondria, and the cytosolic fraction were added to the measurement media at concentrations of 2 mg dry wt/ml, 1 mg protein/ml, and 0.1 mg protein/ml, respectively. The measurement media also contained 1 mM NAD(P)H, 5 μg/ml superoxide dismutase, 10 μM cytochrome *c*, and 1 mM cyanide. Juglone was added at concentrations of 0.1 mM (cells) or 0.05 mM (mitochondria and the cytosolic fraction). Figures along curves *1*, *2*, and *3* indicate the rate of oxygen consumption in, respectively, nmol O_2/(min mg dry cells), nmol O_2/(min mg protein), and nmol O_2/(min mg protein). Measurement conditions are described in the Materials and Methods section.

Cell respiration was measured at 20–22°C using an LP-7 polarograph (Czech Republic) equipped with a Clark-type oxygen electrode.

To obtain mitochondria and the cytosolic fraction of fungal cells, the mycelium was disintegrated with glass beads [13] or using a French press [10].

Glutathione reductase (GR) was assayed by measuring the decline in the NADPH concentration in the presence of oxidized glutathione at 340 nm [14].

Glucose-6-phosphate (G-6-P) dehydrogenase was assayed by measuring the reduction of $NADP^+$ at 340 nm [15].

The formation of superoxide radicals was determined through the reduction of cytochrome *c* measured at 550 nm relative to 600 nm. The reaction mixture contained 0.1 mM EDTA, 1 mM NADH or NADPH, and 10 μM cytochrome *c* in a 20 mM Tris–HCl buffer (pH 7.0).

Spectral measurements were carried out using a Shimadzu UV-160 spectrophotometer (Japan).

The reagents used in the work were a 3% solution of H_2O_2 of domestic production, cytochrome *c* purchased from Sigma, and PMSF and juglone purchased from Serva.

RESULTS AND DISCUSSION

In a previous work [10], the treatment of the fungus *F. decemcellulare* with nonlethal concentrations of hydrogen peroxide and juglone was found to induce the resistance of fungal cells to lethal concentrations of these compounds. In those experiments, juglone (5-hydroxy-1,4-naphthoquinone) was used as a natural superoxide radical–generating agent.

Figure 1 illustrates the effect of juglone on the consumption of oxygen by intact fungal cells, mitochondria, and the cytosolic fraction of the cells. As can be seen from this figure, juglone at a concentration of 0.1 mM relieved the inhibition of cell respiration by 1 mm cyanide (curve *1*). As is evident from Fig. 1, curve *2*, juglone activated cell respiration probably due to its ability to accept electrons from the NADH dehydrogenase of mitochondria located on the outer side of the mitochondrial membrane and to transfer them to oxygen round the respiratory chain. The results presented in Fig. 1, curve *3* indicate that juglone can also accept electrons from cytosolic NADH- and NADPH-dependent enzymes, presumably diaphorases. The reduction of cytochrome *c* by NAD(P)H in the presence of juglone and the cytosolic fraction of cells (Fig. 1, curve *4*) suggests that juglone reduces oxygen with the formation of superoxide radicals. This suggestion is con-

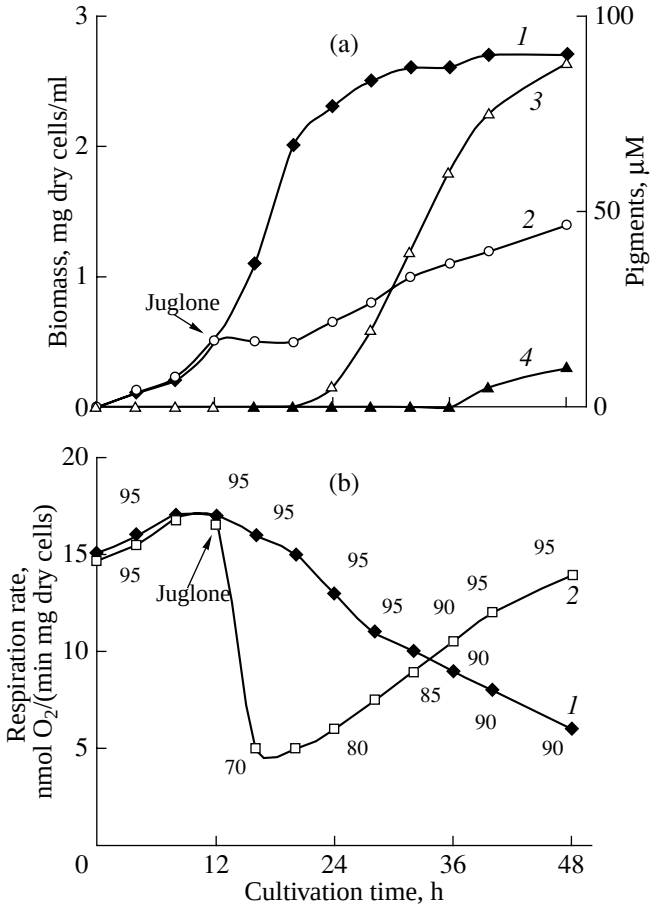

Fig. 2. The effect of 0.1 mM juglone on (a) the growth and naphthoquinone synthesis and (b) the respiration of *F. decemcellulare* cells. Panel a: (*1*) the growth of the control culture; (*2*) the concentration of naphthoquinones in the control culture; (*3*) culture growth after the addition of juglone at the instant indicated by the arrow; and (*4*) the concentration of naphthoquinones in the culture grown in the presence of juglone. Panel b: the respiration of (*1*) the control cells and (*2*) cells exposed to juglone. Figures along the curves indicate the sensitivity of cell respiration to 1 mM cyanide, expressed as a percent.

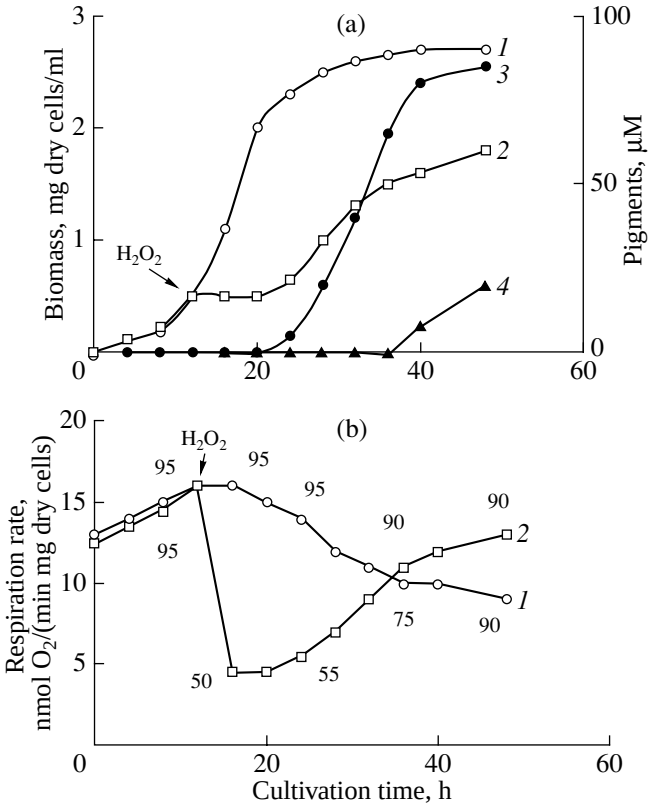

Fig. 3. The effect of 0.25 mM hydrogen peroxide on (a) the growth and naphthoquinone synthesis and (b) the respiration of *F. decemcellulare* cells. Panel a: (*1*) the growth of the control culture; (*2*) the concentration of naphthoquinones in the control culture; (*3*) culture growth after the addition of hydrogen peroxide at the instant indicated by the arrow; and (*4*) the concentration of naphthoquinones in the culture grown in the presence of hydrogen peroxide. Panel b: the respiration of (*1*) the control cells and (*2*) cells exposed to H_2O_2. Figures along the curves indicate the sensitivity of cell respiration to 1 mM cyanide, expressed as a percent.

firmed by the fact that superoxide dismutase completely inhibits the reduction of cytochrome *c* (curve *4*).

Therefore, when present in fungal cells, juglone can generate superoxide radicals and thus diminish the cellular pool of reduced pyridine nucleotides, which are especially necessary to the cells exposed to oxidative stress. Generally, the fungus *F. decemcellulare* is able to synthesize more than ten naphthoquinone pigments [13], which are known to possess biological, particularly phytotoxic, activity [16]. Naphthoquinones reduce the resistance of plants to phytopathogenic fungi and, hence, promote fungal virulence. This prompted us to study the features of naphthoquinone synthesis and some other physiological characteristics of the fungus exposed to oxidative stress (i.e., to treatment with juglone).

Figures 2a and 2b show the effect of juglone on the growth, respiration, and naphthoquinone synthesis in the fungus. Juglone was added to the exponential-phase culture at the moment indicated by the arrow alongside of curves *2*. As can be seen from curve *2* in Fig. 2a, the addition of 0.1 mM juglone arrested the growth of the fungus. After about 12 h, the growth resumed; however, the biomass accumulated in the presence of juglone was considerably lower than in its absence (Fig. 2a, curve *1*). Along with the inhibition of fungal growth, juglone considerably inhibited the synthesis of naphthoquinone pigments, which appeared in the culture liquid after 40 h of cultivation in the presence of juglone (Fig. 2a, curve *4*), and as soon as after 24 h of cultivation in its absence (Fig. 2a, curve *3*). After 48 h of cultivation, the concentration of naphthoquinones in the culture grown in the presence of juglone was considerably lower than in the control culture (cf. curves *3* and *4*).

Table 1. The effect of H_2O_2 and juglone on the biomass and naphthoquinone pigments in the culture liquid of the fungus *F. decemcellulare*

Oxidant, mM	Biomass (mg/ml) and pigments (μM)					
	12 h		24 h		36 h	
	biomass	pigments	biomass	pigments	biomass	pigments
Control	2.3	5	2.3	40	2.7	85
H_2O_2, 0.1	0.9	0	1.4	0	2.1	35
H_2O_2, 0.25	0.65	0	1.0	0	1.8	20
H_2O_2, 0.5	0.5	0	0.5	0	1.0	0
H_2O_2, 1.0	0.5	0	0.5	0	0.5	0
Juglone, 0.05	0.9	0	1.2	0	1.9	55
Juglone, 0.1	0.65	0	0.9	0	1.4	10
Juglone, 0.25	0.5	0	0.5	0	0.9	0
Juglone, 0.5	0.5	0	0.5	0	0.5	0

Note: The oxidants were added to the exponential-phase 12-h-old culture (see Fig. 1). Biomass and pigment accumulation was determined 12, 24, and 36 h after oxidant addition.

Table 2. Cell respiration and its sensitivity to cyanide and benzohydroxamic acid (BHA) after the exposure of the *F. decemcellulare* cells to different concentrations of hydrogen peroxide and juglone

Oxidant, mM	Cell respiration, nmol O_2/(min mg dry cells)								
	12 h			24 h			36 h		
	None	KCN	KCN + BHA	None	KCN	KCN + BHA	None	KCN	KCN + BHA
Control	16	1.5	1.0	14	1.4	1.0	9	1.3	0.8
H_2O_2, 0.1	7.5	1.5	0.5	10	1.5	1.0	16	1.0	0.6
H_2O_2, 0.25	4.5	2.5	0.6	9.5	2.3	0.7	13	1.7	0.7
H_2O_2, 0.5	2.1	1.1	0.6	2.2	1.0	0.7	3.9	1.1	0.7
H_2O_2, 1.0	1.0	1.0	0.9	1.0	1.1	1.0	1.2	1.0	0.9
Juglone, 0.05	7.26	1.2	0.7	9.6	1.4	0.9	15.1	1.7	1.0
Juglone, 0.1	5.3	3.7	0.9	7.1	1.3	0.9	12.5	1.6	1.0
Juglone, 0.25	1.0	1.0	0.7	0	–	–	0	–	
Juglone, 0.5	0	–	–	0	–	–	0	–	–

Note: The oxidants were added to the exponential-phase 12-h-old culture (see Fig. 1). Respiration was measured using cells washed with 10 mM phosphate buffer. KCN and BHA were used at concentrations of 1 and 5 mM, respectively. "–" stands for "not determined."

The addition of 0.1 mM juglone to the exponential-phase *F. decemcellulare* culture led to a more than threefold decrease in the cell respiration (Fig. 2b, curve *2*). After the next 12 h of cultivation, the respiration of fungal cells began to increase concurrently with the culture growth. By the 48 h of cultivation, the respiration of the cells grown in the presence of juglone amounted to 80% of the maximum respiratory activity of the control 12-h-old cells (Fig. 2b, curve *1*).

In the absence of juglone, the respiration of fungal cells was sensitive to cyanide by 90–95% (Fig. 2b, curve *1*). The addition of juglone resulted in a decrease in the cyanide-sensitivity of cell respiration from 95 to 70% (Fig. 2b, curve *2*). As the growth of the fungus resumed, the sensitivity of the respiration to cyanide gradually increased to 95%.

Figures 3a and 3b show the effect of hydrogen peroxide on cell respiration and naphthoquinone synthesis. The addition of 0.25 mM H_2O_2 to the growing culture (this instant is indicated in the figures by the arrows) arrested the growth of the fungus over a period of about 12 h (Fig. 3a, curve *2*). Then the growth resumed. The formation of naphthoquinone pigments in a detectable amount was observed after 40 h of cultivation (Fig. 3a, curve *4*). By the 48th h of cultivation, the concentration of naphthoquinones in the culture grown in the presence of hydrogen peroxide amounted to 30% of their concentration in the control culture (Fig. 3a, curve *3*).

Table 3. Activities of glucose-6-phosphate dehydrogenase and glutathione reductase in the *F. decemcellulare* cells of different age exposed to different concentrations of hydrogen peroxide and juglone

Treatment with	Growth phase	Treatment time, h	Enzymatic activity, μmol NADPH/(min mg protein)*	
			glucose-6-phosphate dehydrogenase	glutathione reductase
Control	Exponential phase	0	28.5	32.6
Control	Stationary phase	0	170.0	151.6
0.25 mM H_2O_2	Exponential phase	1	50.1	47.8
		4	86.3	90.0
		12	56.3	66.3
0.1 mM juglone	Exponential phase	1	60.8	55.1
		4	190.4	160.1
		12	186.5	150.7

* Data are the means of 3–4 replicated measurements. Data scatter did not exceed 10–15%.

The addition of hydrogen peroxide to the culture noticeably inhibited cell respiration (Fig. 3b, curve *2*) and lowered the degree of its sensitivity to cyanide from 95 to 50%. After the next 12 h of cultivation, the cell respiration and its sensitivity to cyanide tended to increase to reach the values typical of the control cells (Fig. 3b, curve *1*).

Data on the effect of different concentrations of hydrogen peroxide and juglone on some relevant physiological parameters of the fungus are summarized in Tables 1 and 2. The increase in the H_2O_2 concentration to 0.5 mM led to a stronger inhibition of fungal growth than in the case shown in Fig. 3. Hydrogen peroxide at a concentration of 1 mM completely inhibited the growth and naphthoquinone synthesis. The rate of fungal respiration and its sensitivity to cyanide also decreased (Table 2). The inhibition of the cyanide-resistant respiration by benzohydroxamic acid (BHA) indicates that this respiration is due to the functioning of cyanide-resistant alternative oxidase. Hydrogen peroxide at a concentration of 0.1 mM caused only a transient inhibition of fungal growth and naphthoquinone synthesis.

Like hydrogen peroxide, juglone at elevated concentrations of 0.25 and 0.5 mM inhibited, either partially or completely, growth, cell respiration, and naphthoquinone synthesis (Tables 1 and 2).

The results presented in this paper show that hydrogen peroxide and juglone affect the respiratory chain of the fungus, eventually causing its death. Experiments with animal cells and subcellular structures showed that active oxygen species impair mitochondria and induce peroxidation of cardiolipin, which is necessary for cytochrome *c* oxidase to be active [17]. The observation that the yeast *Saccharomyces cerevisiae* cells with impaired mitochondria are distinguished by increased sensitivity to hydrogen peroxide [18] is also indicative of the great importance of mitochondria in providing for cell resistance to oxidative stress.

The role of glutathione reductase and G-6-P dehydrogenase as enzymes involved in the protective mechanisms of yeasts against oxidative stress has been well recognized [19, 20]. The latter enzyme is largely responsible for the regeneration of NADPH necessary for the reduction of oxidized glutathione. In our experiments, the addition of hydrogen peroxide and juglone at nonlethal concentrations to fungal cells led to an increase in their glutathione reductase and G-6-P dehydrogenase activities, which began 60 min after the addition. The maximum (about threefold) increase in the activities was observed 4 h after the addition of the oxidants. After the next 8 h of incubation in the presence of hydrogen peroxide, the activity of both enzymes tended to slow down. Conversely, the activity of the enzymes in the presence of juglone declined insignificantly, probably due to the fact that this compound not only generates superoxide radicals (like H_2O_2) but also decreases the intracellular pool of reduced pyridine nucleotides. The glutathione reductase and G-6-P dehydrogenase activities of the stationary-phase (36-h-old) *F. decemcellulare* cells were more than fivefold higher that those of exponential-phase cells (data not shown), being comparable with the levels of these activities in juglone-treated cells.

To conclude, glutathione reductase and glucose-6-phosphate dehydrogenase play an important role in the protection of phytopathogenic fungi against oxidative stress.

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