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EXPERIMENTAL ARTICLES

Adaptation of the Phytopathogenic Fungus *Fusarium decemcellulare* to Oxidative Stress

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Abstract—The adaptive response of the phytopathogenic fungus *Fusarium decemcellulare* to the oxidative stress induced by hydrogen peroxide and juglone (5-hydroxy-1,4-naphthoquinone) was studied. At concentrations higher than 1 mM, H_2O_2 and juglone completely inhibited the growth of the fungus. The 60-min pretreatment of logarithmic-phase cells with nonlethal concentrations of H_2O_2 (0.25 mM) and juglone (0.1 mM) led to the development of a resistance to high concentrations of these oxidants. The stationary-phase cells were found to be more resistant to the oxidants than the logarithmic-phase cells. The adaptation of fungal cells to H_2O_2 and juglone was associated with an increase in the activity of cellular catalase and superoxide dismutase, the main enzymes involved in the defense against oxidative stress.

Key words: fungus, oxidative stress, hydrogen peroxide, juglone, survival, adaptation mechanism, catalase, superoxide dismutase.

It is known that active oxygen species (AOSs), including hydrogen peroxide (H_2O_2), the superoxide radical ($O_2^{\bullet}$), and the hydroxyl ion ($OH^{\bullet}$), are toxic to microbial cells. The toxicity of these species is determined by their ability to oxidize proteins and lipids, thus inflicting damage on various cellular structures, as well as by their ability to induce DNA and RNA breaks [1, 2].

In response to the action of AOSs, many organisms activate the synthesis of some enzymes (catalase, superoxide dismutase (SOD), and others) and the production of reduced metabolites capable of detoxifying AOSs (reduced glutathione, NAD(P)H, etc.) [3, 4].

One of the primary responses of plants to phytopathogenic invasion is the activation of H_2O_2-generating processes [5–7]. In addition, many plants produce defense autooxidizable naphthoquinone pigments [8], which oxidize NAD(P)H in plant and phytopathogenic cells with the formation of superoxide radicals [9] toxic to phytopathogens. Therefore, for an efficient invasion of plants, phytopathogenic fungi require a mechanism for the detoxication of AOSs.

Relevant data concerning phytopathogenic fungi are scarce.

The aim of the present work was to study the survival of *Fusarium decemcellulare* under oxidative stress, with special emphasis on changes in the catalase and superoxide dismutase activities of this phytopathogenic fungus in response to hydrogen peroxide and juglone in the first stages of adaptation to these oxidants.

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 [10] containing 1% glucose as the source of carbon and energy.

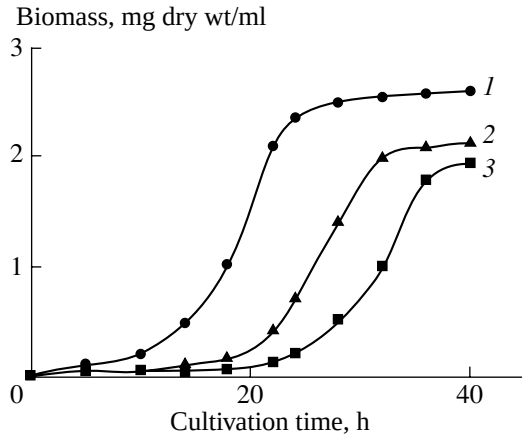

Fig. 1. Growth of *F. decemcellulare* (*1*) without additions and in the presence of (*2*) 0.25 mM hydrogen peroxide or (*3*) 0.1 mM juglone.

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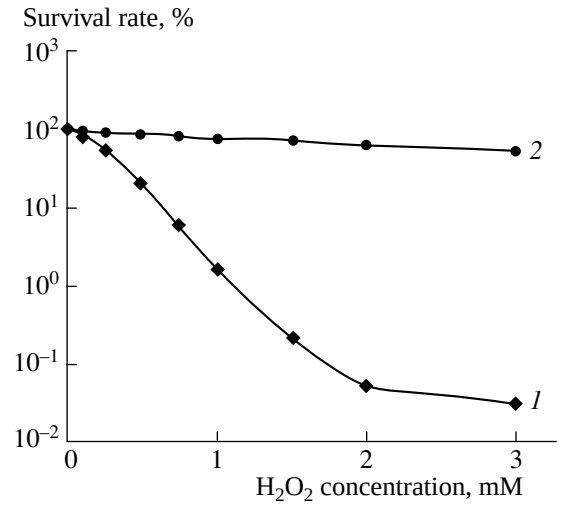

Fig. 2. Hydrogen peroxide resistance of the exponential-phase *F. decemcellulare* cells, either (*1*) untreated or (*2*) pretreated with a nonlethal concentration (0.25 mM) of H_2O_2 for 60 min.

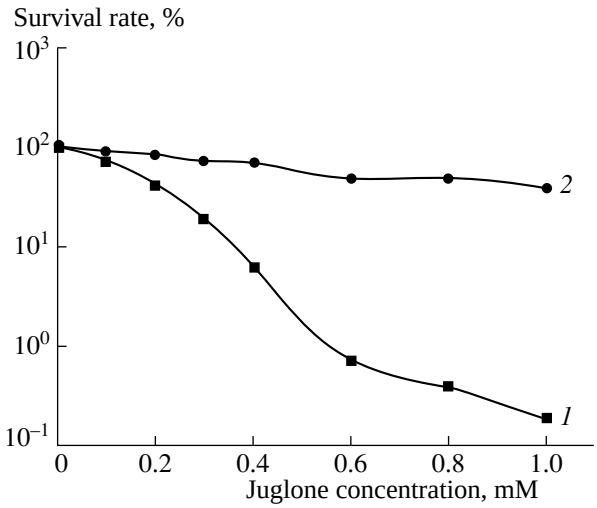

Fig. 3. Juglone resistance of the exponential-phase *F. decemcellulare* cells, either (*1*) untreated or (*2*) pretreated with a nonlethal concentration (0.1 mM) of juglone for 60 min.

The material for inoculum was one-day mycelium. Growth was monitored by measuring the dry weight of fungal biomass.

To evaluate fungal survival under oxidative stress, 10-ml exponential-phase (10–12 h of growth) or stationary-phase (36 h of growth) cultures grown in 100-ml flasks were aseptically supplemented with 0.25 mM H_2O_2 or 0.1 mM juglone and cultivated for 60 min. Cells were then harvested by centrifugation, washed with sterile distilled water, resuspended in the same volume (10 ml) of the growth medium with elevated concentrations of the oxidants, and incubated at 29°C on the shaker for 60 min. To assay cell survival, cell samples were taken at 20-min intervals, diluted with the growth medium, and plated on agar medium. The number of colonies grown on agar plates was determined after incubating them at 29°C for 60–72 h. The data presented are the averages of triplicate experiments.

Extracts for the assay of catalase and SOD were prepared from the cells adapted to 0.25 mM H_2O_2 or 0.1 mM juglone. Exponential-phase cells were incubated for 60 min with the oxidants taken at the concentrations indicated, washed with distilled water, resuspended in 50 mM phosphate buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted in a French press. The homogenate was centrifuged at 105000*g* for 60 min. The pellet was discarded and the supernatant was used for the enzyme assay.

The activity of catalase was determined from the decrease in concentration of H_2O_2 estimated by measuring the optical density of the reaction mixture at 246 nm [11]. One unit of activity was defined as the amount of enzyme decomposing 1 μmol of H_2O_2 per minute.

The activity of SOD was determined by measuring the degree of inhibition of the reduction of 10 μM cytochrome *c* by the superoxide radical in 50 mM Tris–HCl (pH 7.5) containing 0.5 mM xanthine, 0.5 E xanthine oxidase, 1 mM EDTA, and 5 mM $MgCl_2$ [12]. One unit of activity was defined as the amount of enzyme required for the 50% inhibition of reduction of cytochrome *c* in this coupled system.

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

The reagents used were a 3% solution of H_2O_2 (local produced), cytochrome *c* purchased from Sigma, and PMSF, juglone, xanthine, and xanthine oxidase purchased from Serva.

RESULTS

Juglone is an autoxidizable agent capable of accepting electrons from reduced flavine-containing NAD(P)-dependent enzymes (DT-diaphorase and others), with the formation of superoxide radicals. Figure 1 illustrates the effect of 0.25 mM H_2O_2 and 0.1 mM juglone (curves *2* and *3*, respectively) on fungal growth. As can be seen from this figure, either oxidant considerably lengthened the lag phase of the fungus compared to the control (curve *1*) so that noticeable growth began only after 20–24 h of incubation. At a concentration of 1 mM, juglone and hydrogen peroxide completely inhibited fungal growth.

Data on the effect of the oxidants on the survival of exponential-phase *F. decemcellulare* cells are presented in Fig. 2. At concentrations higher than 2 mM, hydrogen peroxide diminished the survival rate of the fungus to values lower than 0.1% (curve *1*). The pretreatment of fungal cells with a nonlethal concentration

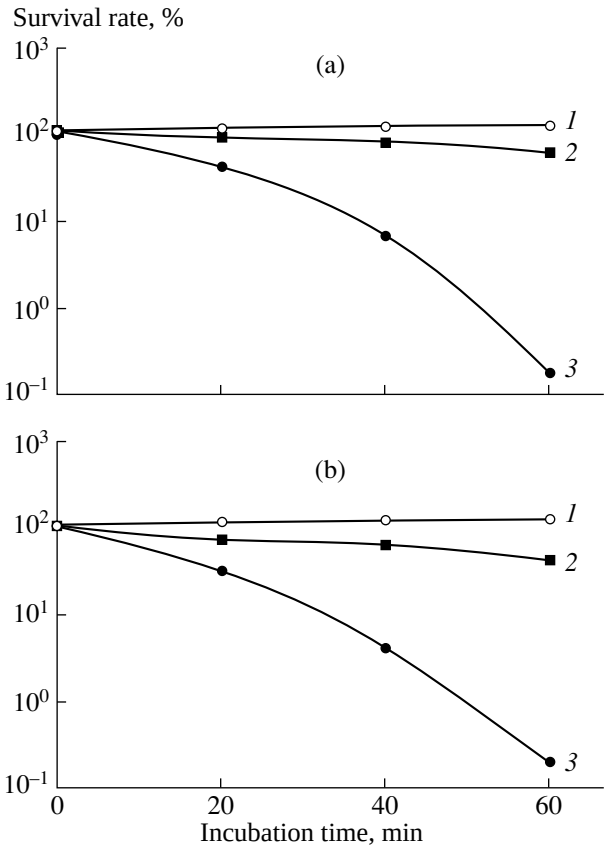

Fig. 4. Resistance to (a) 2 mM hydrogen peroxide and (b) 1 mM juglone of (*3*) exponential phase and (*2*) stationary phase *F. decemcellulare* cells. Curves *1* show the survival of fungal cells in the absence of the oxidants.

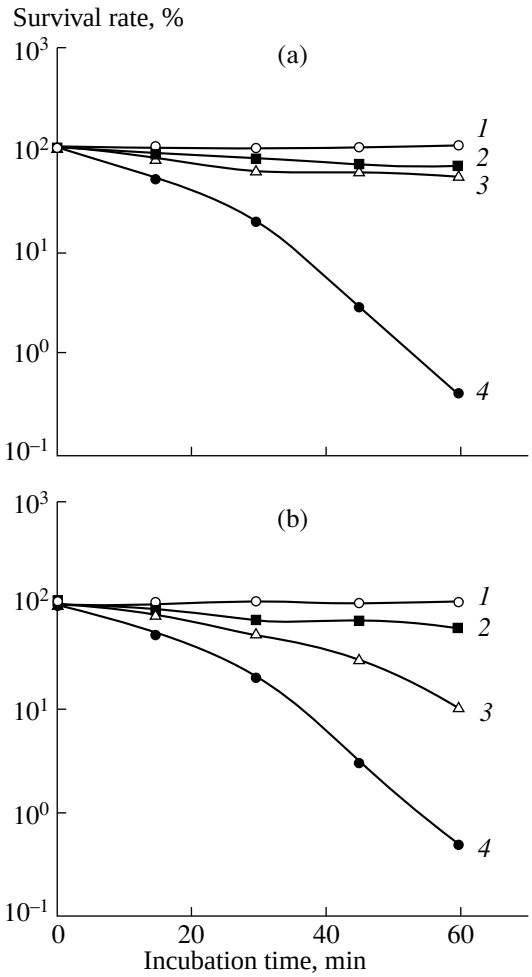

Fig. 5. Resistance to (a) 2 mM hydrogen peroxide and (b) 1 mM juglone of the exponential phase *F. decemcellulare* cells pretreated for 60 min with (*2*) 0.25 mM H_2O_2 or (*3*) 0.1 mM juglone. Curves *1* show the survival of fungal cells in the absence of the oxidants. Curves *4* show the survival of nonadapted cells.

of H_2O_2 (0.25 mM) led to a considerable increase in the survival rate, even in the presence of 3 mM H_2O_2 (curve *2*).

Similarly, the pretreatment of the exponential phase cells with a nonlethal concentration of juglone (0.1 mM) resulted in the development of a resistance to lethal concentrations of this oxidant (Fig. 3).

Figure 4 shows the dependence of the survival of *F. decemcellulare* cells on the growth phase. As can be seen from this figure, the stationary phase cells exhibited a higher resistance to H_2O_2 (Fig. 4a) and juglone (Fig. 4b) than the exponential phase cells.

The adaptive effect of hydrogen peroxide and juglone was not specific. Indeed, the pretreatment of exponential phase cells with 0.25 mM H_2O_2 led to the development of a resistance to lethal concentrations of juglone (Fig. 5a, curve *2*). On the other hand, pretreatment of the exponential-phase cells with 0.1 mM juglone made them resistant to lethal concentrations of hydrogen peroxide (Fig. 5b, curve *2*). It should be noted that the resistance of H_2O_2-pretreated cells to juglone was slightly lower than that of the juglone-pretreated cells.

In our attempts to elucidate the possible mechanism of the enhanced resistance of fungal cells to oxidants, we measured the activities of catalase and superoxide dismutase in exponential phase cells before and after their treatment with oxidants (see table). As can be seen from the table, the activity of the catalase rose 3.6 times with the pretreatment of the fungal cells with H_2O_2, while the activity of SOD increased insignificantly. The pretreatment of cells with juglone resulted in an increase in catalase activity by 1.5 times and a greater than 3-fold increase in SOD activity.

The activities of these enzymes in the *F. decemcellulare* stationary phase cells were about two times higher than in the exponential phase cells. This correlates with the enhanced resistance of the stationary phase cells to hydrogen peroxide and juglone.

DISCUSSION

The exponential phase *F. decemcellulare* cells are relatively sensitive to hydrogen peroxide and juglone

and lethal concentrations of these oxidants for this fungus are 1 mM. According to data available in the literature, microorganisms differ in their sensitivity to hydrogen peroxide. For instance, the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* are sensitive to hydrogen peroxide, and a lethal concentration of H_2O_2 for these microorganisms is about 2 mM [13, 15]. On the other hand, about 1% of *Streptomyces coelicolor* cells survive in 20 mM H_2O_2 over a period of 20 min [14], *Schizosaccharomyces pombe* is tolerant to 10 mM H_2O_2 and survives at a rate of 10% in 40 mM H_2O_2 [16], and *Candida albicans* is tolerant to 50 mM H_2O_2 [17].

In our experiments, pretreatment of the exponential phase *F. decemcellulare* cells with low concentrations of H_2O_2 and juglone led to a noticeable increase in the cell resistance to these oxidants (Figs. 2 and 3). This suggests that the fungus can adapt to oxidative stress.

The adaptation of *F. decemcellulare* cells to oxidative stress is most likely related to the induction of the synthesis of protective enzymes (catalase in the case of pretreatment with H_2O_2 and both catalase and SOD in the case of pretreatment with juglone). It should be noted that the induction of both protective enzymes by juglone (see table) can explain the higher degree of resistance of juglone-adapted fungal cells to either oxidant as compared to H_2O_2-adapted cells (Fig. 5). The observation that juglone induces the synthesis of catalase and makes cells resistant to hydrogen peroxide is attributable to the fact that this pigment generates superoxide radicals, which dismutate to hydrogen peroxide.

Our observation that the stationary phase *F. decemcellulare* cells are more resistant to the oxidants than the exponential phase cells (Fig. 4) is in agreement with the relevant data obtained for *Schi. pombe* [16, 17] and *S. cerevisiae* [18]. The higher activities of catalase and superoxide dismutase in the stationary phase cells (see table) explain their enhanced tolerance to the oxidants.

Li *et al*. reported that stationary phase yeast cells are more resistant to stresses, including heat shock, than exponential phase cells [16]. The pretreatment of yeast cells with hydrogen peroxide made them resistant to heat shock, high ethanol concentrations, and other stresses [18]. On the other hand, yeast cells subjected to heat shock were found to develop a resistance to hydrogen peroxide [15].

The molecular mechanism responsible for the adaptive response of microorganisms to oxidative stress is as yet unknown. Taking into account that one stress can induce tolerance to other stresses, one can suggest the existence of a common signal that triggers protective mechanisms in microbial cells.

In yeasts, oxidative and other stresses induce the transcription of the catalase gene mediated by specific regulatory sequences (the so-called stress response elements) [19]. Depending on the conditions, gene transcription is regulated by cAMP-dependent or cAMP-independent mechanisms [20]. This suggests that factors influencing the cellular cAMP pool may induce the adaptive response of cells. This problem is considered in an accompanying paper.

Activities of catalase and superoxide dismutase (SOD) in *F. decemcellulare* cells from different growth phases and the effect of pretreatment with hydrogen peroxide and juglone

Cells	Growth phase	Catalase, U/mg protein	SOD, U*/mg protein
Untreated	Exponential	38 ± 5	51 ± 5
Incubated with 0.25 mM H_2O_2 for 60 min	Exponential	140 ± 8	74 ± 7
Incubated with 0.1 mM juglone for 60 min	Exponential	45 ± 6	188 ± 14
Untreated	Stationary	78 ± 7	98 ± 11

* The amount of SOD producing 50% inhibition of cytochrome *c* reduction by superoxide radical was taken as the activity unit.

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