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Cyanide-Resistant Alternative Respiration is Strictly Correlated to Intracellular Peroxide Levels in *Acremonium Chrysogenum*

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A strict correlation between the intensity of the cyanide-resistant alternative respiratory pathway and the intracellular peroxide levels in the cephalosporin C producer filamentous fungus *Acremonium chrysogenum* was demonstrated. Intracellular peroxide levels increased in a dose-dependent manner after addition of H_2O_2 to the culture media. A similar phenomenon was observed due to the specific inhibition of catalase by salicylic acid. In both cases, cyanide-resistant respiration was markedly stimulated. On the other hand, both cyanide-resistant respiration and intracellular peroxide levels were effectively suppressed by the lipid peroxyl radical scavenger DL-α-tocopherol, which breaks lipid peroxidation chains effectively. Our findings firmly supported the assumption that there is a connection between the intracellular peroxide levels and the intensity of the alternative respiratory pathway in fungi.

Keywords: alternative respiration, *Acremonium chrysogenum*, peroxide, salicylic acid, DL-α-tocopherol, catalase

INTRODUCTION

Plants and some other organisms including fungi and protists have been reported to posses a cyanide-resistant, non-phosphorylating and, therefore, energy-dissipating alternative respiratory route in their inner mitochondrial membrane in addition to the phosphorylating, cytochrome-dependent respiratory pathway.[1,2] In plants, the alternative respiration is considered to accomplish different physiological functions. At first, it contributes to the thermogenesis[3] and the maintenance of the mitochondrial electron transport at low temperatures.[4] Moreover, it may also take part in the stabilisation of the redox state of the ubiquinone pool in the inner mitochondrial membrane[5,6], thereby preventing the intracellular accumulation of harmful free radicals.[7–11] It is worth noting that the inhibition of the cytochrome-dependent respiratory pathway normally results in increased reactive oxygen concentrations and, concomitantly, in enhanced cyanide-resistant respiration.[7,11,12] The addition of exogenous hydrogen peroxide to *Petunia hybrida* cells was also shown to provoke the induction of the alternative respiratory pathway.[8]

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There are numerous data indicating the existence of alternative respiration in different kinds of fungi. Despite some marked differences to plants in their regulatory features[13], the fungal alternative pathways also respond to inhibition of the cytochrome-dependent respiration[14–19] and exogenous H_2O_2.[18] Therefore, a similar physiological function of the fungal cyanide-resistant alternative respiratory pathways to that of the analogous plant respiratory systems, e.g. participation in the maintenance of the redox state of the mitochondrial membrane and in the protection against reactive oxygen species, can be proposed.

As reported before, the alternative respiratory pathway of the cephalosporin C producer filamentous fungus *Acremonium chrysogenum* was stimulated by increasing oxygen transfer rates, decreasing specific growth rates and by the stimulation of succinic dehydrogenase through the addition of plant oils to the culture media.[20–22] In the present paper we show that the alternative respiration of the fungus was strictly correlated to intracellular peroxide levels adjusted by the addition of either DL-α-tocopherol (vitamin E) and / or H_2O_2 to the culture media or the selective inhibition of the intracellular catalase activity by salicylic acid. Alternative respiration is therefore likely to be an integral part of the antioxidant defence system in fungi as well.

MATERIALS AND METHODS

Strain and cultivation conditions

Acremonium chrysogenum ATCC 46117 strain was grown on an NBS orbital shaker, at 28°C and 200 rpm, as described earlier.[23] The complete fermentation medium[22] was inoculated with 10% 3 day old seed culture, prepared from a medium consisting of 5 g l^{-1} $CaCO_3$, 10 g l^{-1} peptone, 26.8 g l^{-1} yeast extract, and 40 g l^{-1}glucose. Mycelia were grown for 14 h and then were separated by filtration on sintered glass, washed and transferred into a minimal medium supplemented with glucose as a sole carbon source.[24] The starting mycelial dry weight was appr. 4.0 g l^{-1} in each experiment. Reagents (H_2O_2, DL-α-tocopherol and salicylic acid) were supplied to the culture media 3 h after the transferring procedure. Unless otherwise indicated samples were taken at 4 h after the addition of the supplementary components.

Determination of catalase activity

Catalase activity was measured by a photometric method.[25] For intracellular enzyme activity determinations, cell-free extracts were prepared by disruption of 0.1 g (dry cell weight) quantities of mycelia suspended in 10 ml 5 mM phosphate buffer, pH 7.2, in a pre-cooled AB Biox Type X25 X-press (Göteborg, Sweden). Cellular debris was removed by centrifugation (15000 g, 5 min), and the supernatants were used immediately for enzyme assays. In extracellular catalase activity measurements, *A. chrysogenum* cells were separated by centrifugation (5000 g, 2 min) and the supernatants were used further. In both cases, the deleterious effect of *A. chrysogenum* proteases[24] was eliminated by the addition of phenylmethylsulfonyl fluoride (50 µl into 2 ml of sample), dissolved in isopropanol (10 mg ml^{-1}).[26]

Respiratory measurements

Intensities of the cytochrome-dependent and alternative respiratory pathways were measured in a thermostated oxygraphic cell coupled to an analogue recorder.[27] The cytochrome-dependent pathway was inhibited with 1 mM KCN.

Analytical methods

Mycelial growths were followed by recording dry cell weights. A sample of 2 ml medium was filtered under a vacuum through a Sartorius glass wool filter (SM 134). The remaining mycelia were dried in an oven at 80°C.[28]

Specific growth rates were calculated from the time-course changes in dry cell weight within the four hour long period.[29]

Protein concentrations were determined by the dye-binding method[30], using BSA as a standard.

Intracellular peroxide levels were characterised by the spectrofluorimetric determination of 2',7'-dichlorofluorescein (2',7'-DCF) production from 2',7'-dichlorofluorescin diacetate.[31,32] 2',7'-dichlorofluorescin diacetate was dissolved in 95% ethanol and was added to the culture at 11 μM concentration. After incubating further for 1 h, mycelia were separated and resuspended in 1 mM phosphate-buffer, pH 7. Cell-free extracts were prepared by disruption of 0.1 g (dry cell weight) quantities of mycelia in a pre-cooled X-press. Cellular debris was removed by centrifugation (15000 g, 5 min), and the supernatants were immediately used for assays. According to the literature, both the extracellular decomposition and the mycelial absorption of 2',7'-dichlorofluorescin diacetate are negligible and do not interfere with the determinations.[32]

Extracellular H_2O_2 concentrations were determined by a polarographic method using catalase as an auxiliary enzyme.[33]

Glucose consumption was followed by HPLC on a Bio-Rad Aminex HPX-H^+ ion-exchange column, eluted with 5 mM H_2SO_4. Detection was carried out by measuring the refractive index.[34]

Reproducibility

All the data presented here are Means of at least 3 independent experiments. The variations between experiments were estimated by standard deviations (SDs) for each procedure. The SD values were always less than 10 % of the Means. Significances of changes as a function of reagent concentrations were estimated using the Student's *t*-test. In dose-dependence experiments, the cited probability *p* values refer to the differences observed between controls and the highest reagent concentrations.

Chemicals

All chemicals were of analytical grade, and were purchased from Sigma-Aldrich Kft., Budapest, Hungary with the exceptions of 2',7'-dichlorofluorescin diacetate and 2',7'-dichlorofluorescein, which were bought from Eastport Kft., Budapest, Hungary.

RESULTS

Effects of Exogenously Added Hydrogen Peroxide

Growth and glucose consumption rates

A. chrysogenum cells showed a remarkable resistance against oxidative stress generated by exogenous hydrogen peroxide. Mycelia survived the exposure to H_2O_2 at concentrations as high as 200 mM. However, the specific growth rates during the examined period decreased profoundly with increasing H_2O_2 concentrations ($p<0.1\%$), and even became negative above 150 mM levels (Figure 1). Surprisingly, the glucose consumption rates significantly ($p<1\%$) increased with increasing H_2O_2 concentrations (Figure 1). It is worth noting, that the negative growth of the peroxide-treated cultures was only temporary, since leaving them in rotary shaker for an additional 20 hours resulted in renewed biomass production comparable to the control cultures.

Uninhibited and cyanide-resistant respiration

The total, uninhibited respiration rates of H_2O_2-treated cultures were slightly higher than those found in control cultures, meanwhile the activity of the cyanide-insensitive alternative respiration was significantly ($p<0.1\%$) stimulated by H_2O_2 (Figure 1). At 200 mM H_2O_2 concentration the cyanide-resistant respiration comprised more than 70 % of the total oxygen consumption.

As shown in Figure 2, the cyanide-resistant respiration started to increase at 1 h incubation

time after the treatment with 200 mM H_2O_2, increased considerably between 1 and 2 h and reached a plateau between 3 and 4 h of incubation. Very similar time-courses were observed with all the other hydrogen peroxide concentrations tested (data not shown). It is worth noting that any increase in the alternative respiration could be prevented by the addition of 0.7 M cycloheximide at 1 h incubation time.

Intracellular peroxide levels

Intracellular peroxide levels were significantly ($p<1\%$) and dose-dependently higher at 4 h of incubation in H_2O_2-treated cultures than that observed in controls (Figure 1). Moreover, similar to the alternative respiration (Figure 2), the intracellular peroxide levels showed maximum values between 3 and 4 h of incubation (Figure 2).

Extracellular and intracellular catalase activities

As indicated in Figure 3, the spontaneous decomposition of H_2O_2 was negligible for several days in mycelia-free culture medium. On the other hand, the elimination of $H_2O_2$was remarkably fast in the presence of *A. chrysogenum* cells (Figure 3), clearly indicating a potent peroxide-eliminating system in *A. chrysogenum* mycelia. Indeed, we detected high intracellular and extracellular catalase activities which were produced constitutively by the fungus (Figure 3). Data displayed are from measurements made at 4 hours after the addition of H_2O_2, but the same values were measured from samples taken immediately and at 16 hours after the addition of 200 mM H_2O_2, respectively.

Effects of Suppressed Hydrogen-Peroxide Elimination

Addition of salicylic acid to the culture medium

Similar to plant catalases, the intracellular catalase activity was inhibited effectively ($p<1\%$) and in a dose-dependent manner by salicylic acid *in vivo* (Figure 4). Concomitantly, the cyanide-insensitive respiration increased with increasing salicylic acid doses (Figure 4). Salicylic acid also increased the intracellular peroxide concentrations significantly ($p<1\%$) and in a dose-dependent manner (Figure 4), while it exhibited no significant effects on the growth and glucose consumption rates (data not shown).

Effects of Enhanced Hydrogen-Peroxide Elimination

As shown in Figure 5, the free radical scavenger DL-α-tocopherol had a striking effect on the alternative respiration and the intracellular peroxide levels. DL-α-Tocopherol dose-dependently and effectively ($p<0.1\%$) eliminated intracellular peroxides generated by treatment with 200 mM H_2O_2 and concomitantly hindered the alternative respiratory pathway. Added to control cultures in an increasing concentration, intracellular peroxide levels declined until they went down below the lower limit of sensitivity of the 2'7'-DCF method (Figure 5). At the same time, cyanide-resistant respiration also decreased until, at 150 mg l^{-1} DL-α-tocopherol concentration, it could not be detected. That is the intracellular peroxide levels and the intensity of the alternative respiratory pathway seem to be again strictly correlated.

DISCUSSION

The existence of the cyanide-resistant alternative respiratory pathway is known for more than seven decades now[35], yet its regulation is not fully understood. In plants the intensity of the alternative route will depend on the amount of oxidase present[36], on the redox status of the redox-sensitive disulphide bond between neighbouring subunits[37], on the intracellular concentration of organic acids (particularly of

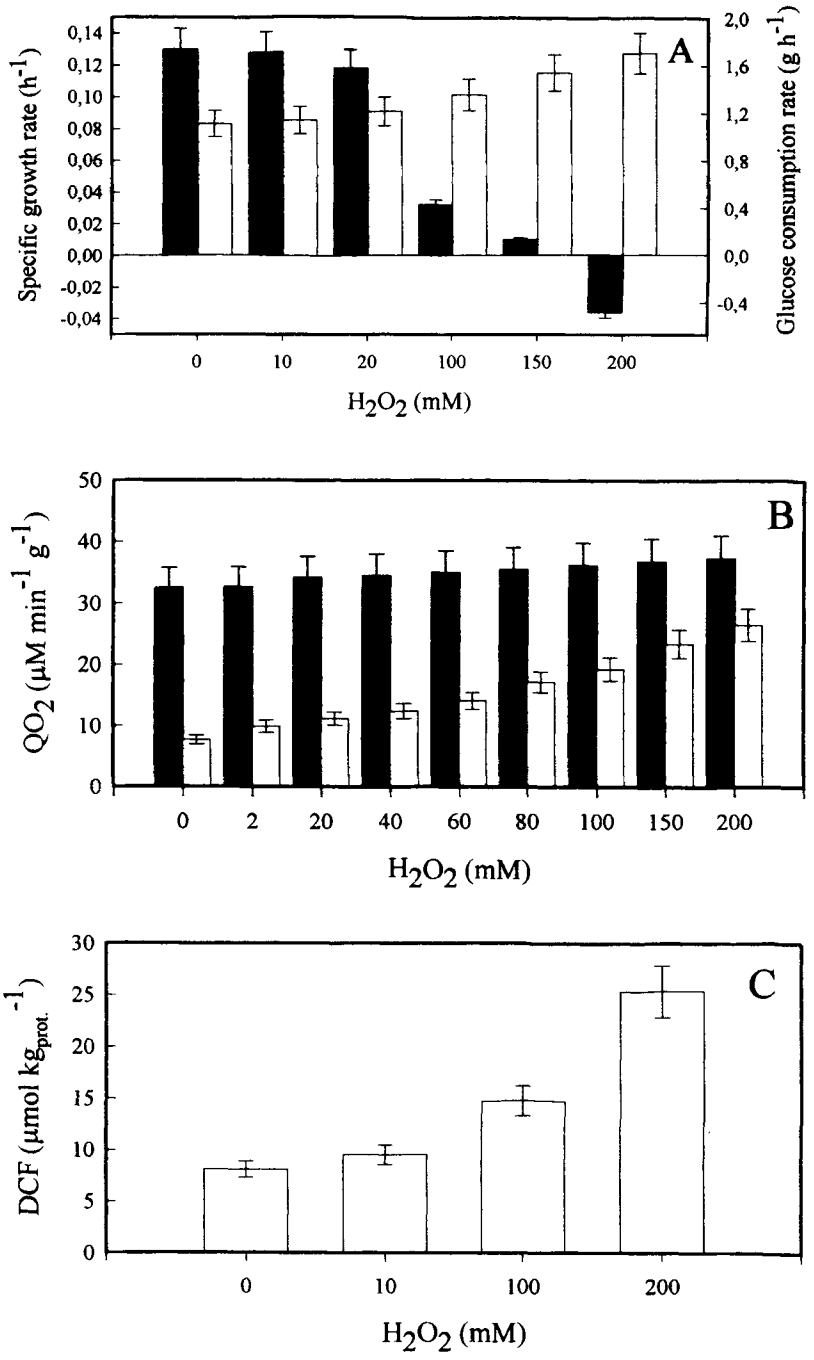

FIGURE 1 Effects of hydrogen-peroxide on *A. chrysogenum* cultures. **Part A**. Specific growth rate (■) and glucose consumption rate (□). **Part B**. Total (■) and cyanide-resistant (□) respiration rates **Part C**. Intracellular peroxide levels at 4 hours after the addition of H_2O_2

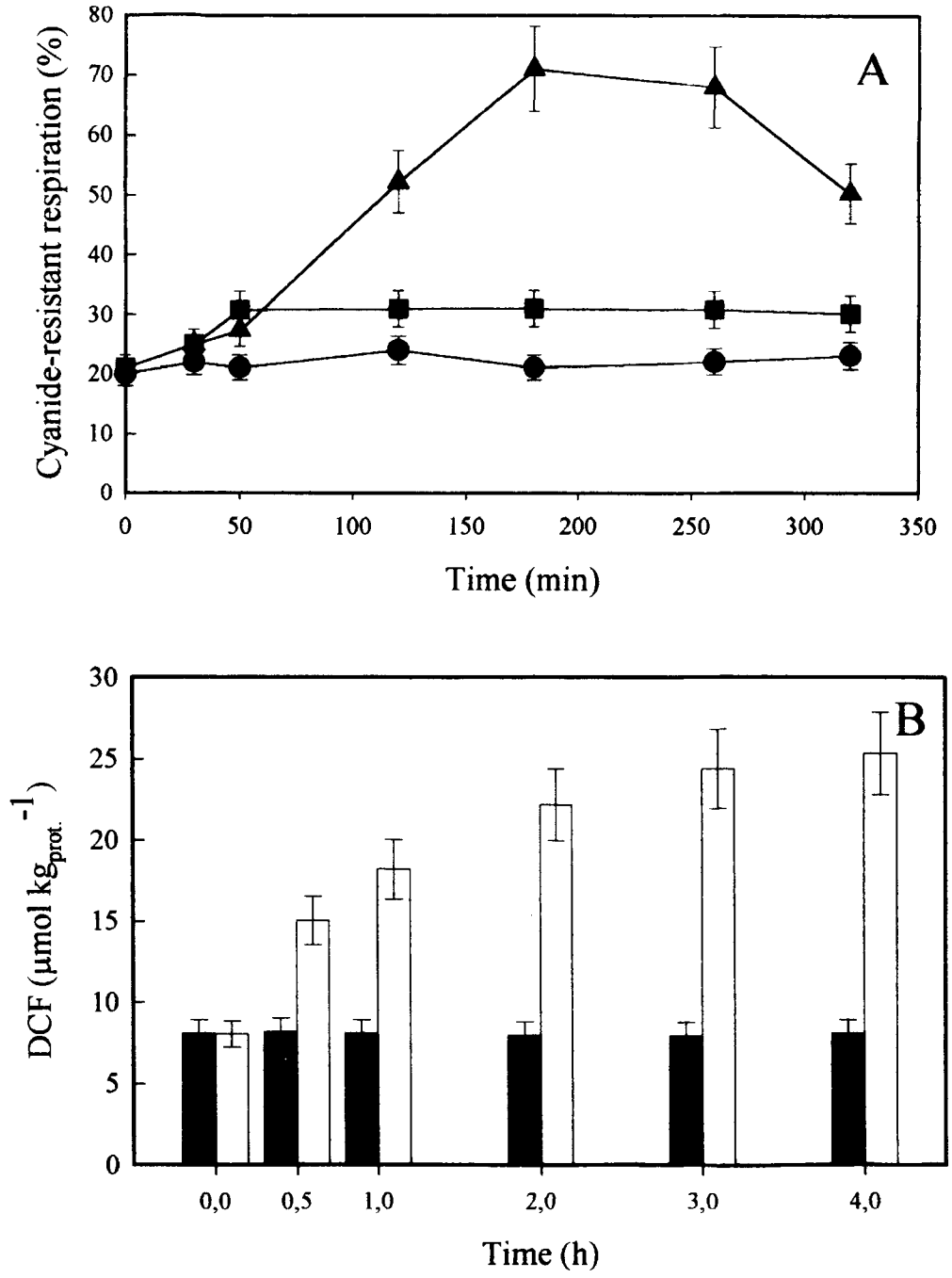

FIGURE 2 Time course of changes following the addition of 200 mM hydrogen-peroxide. **Part A.** Stimulation of the cyanide-resistant respiration (▲), and its repression by 0.7 M cycloheximide (■). • indicates control values. **Part B.** Intracellular peroxide levels. ■ indicates control values

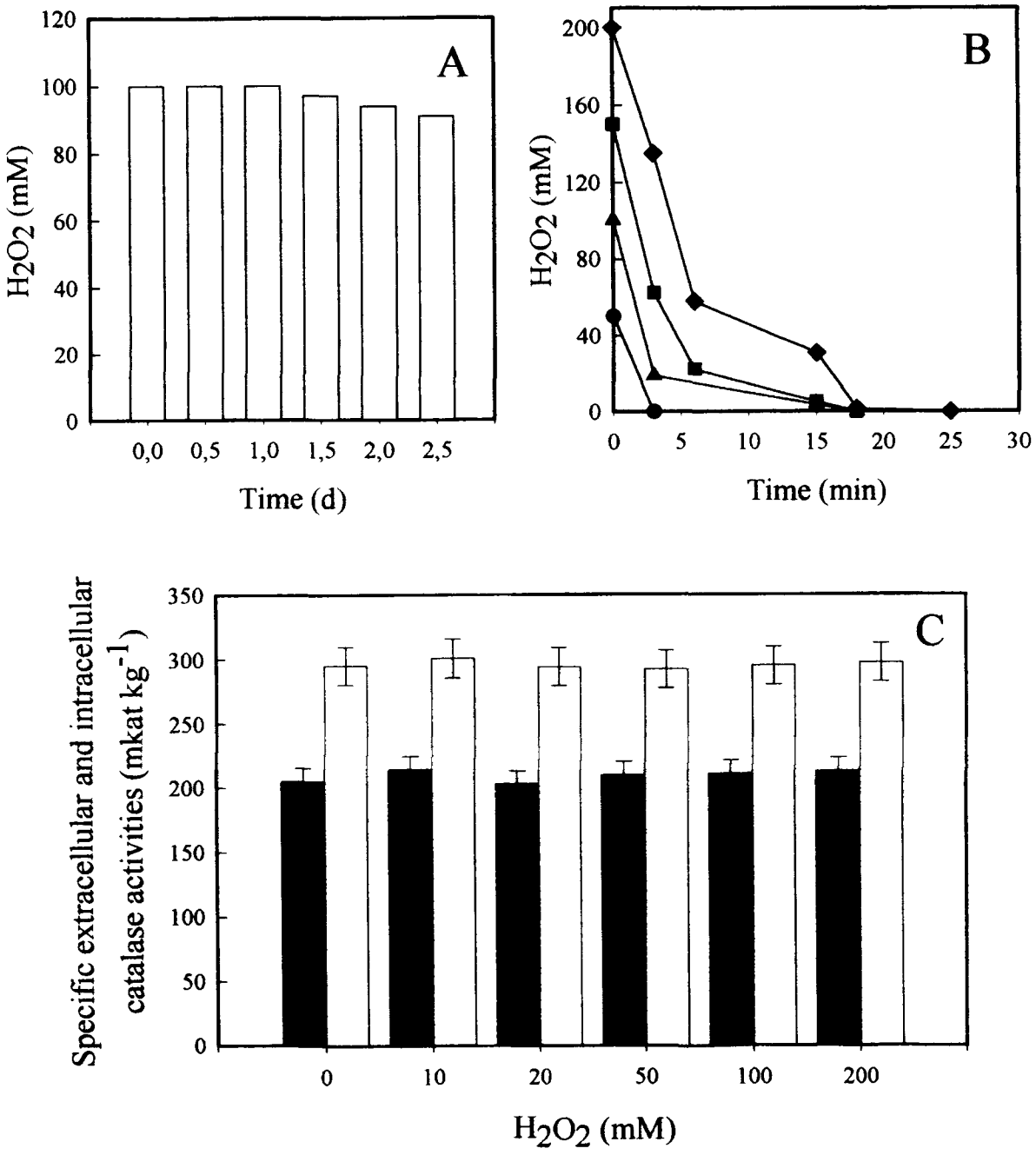

FIGURE 3 Elimination of hydrogen-peroxide and activity of catalases in *A. chrysogenum* cultures. **Part A**. Decomposition of H_2O_2 in the absence of cells. **Part B**. Decomposition of H_2O_2 in the presence of *A. chrysogenum* mycelia. ♦: 200 mM H_2O_2; ■: 150 mM H_2O_2; ▲: 100 mM H_2O_2; •: 50 mM H_2O_2. **Part C**. Intracellular (□) and extracellular (■) catalase activities in *A. chrysogenum* cultures at 4 hours after H_2O_2 treatment

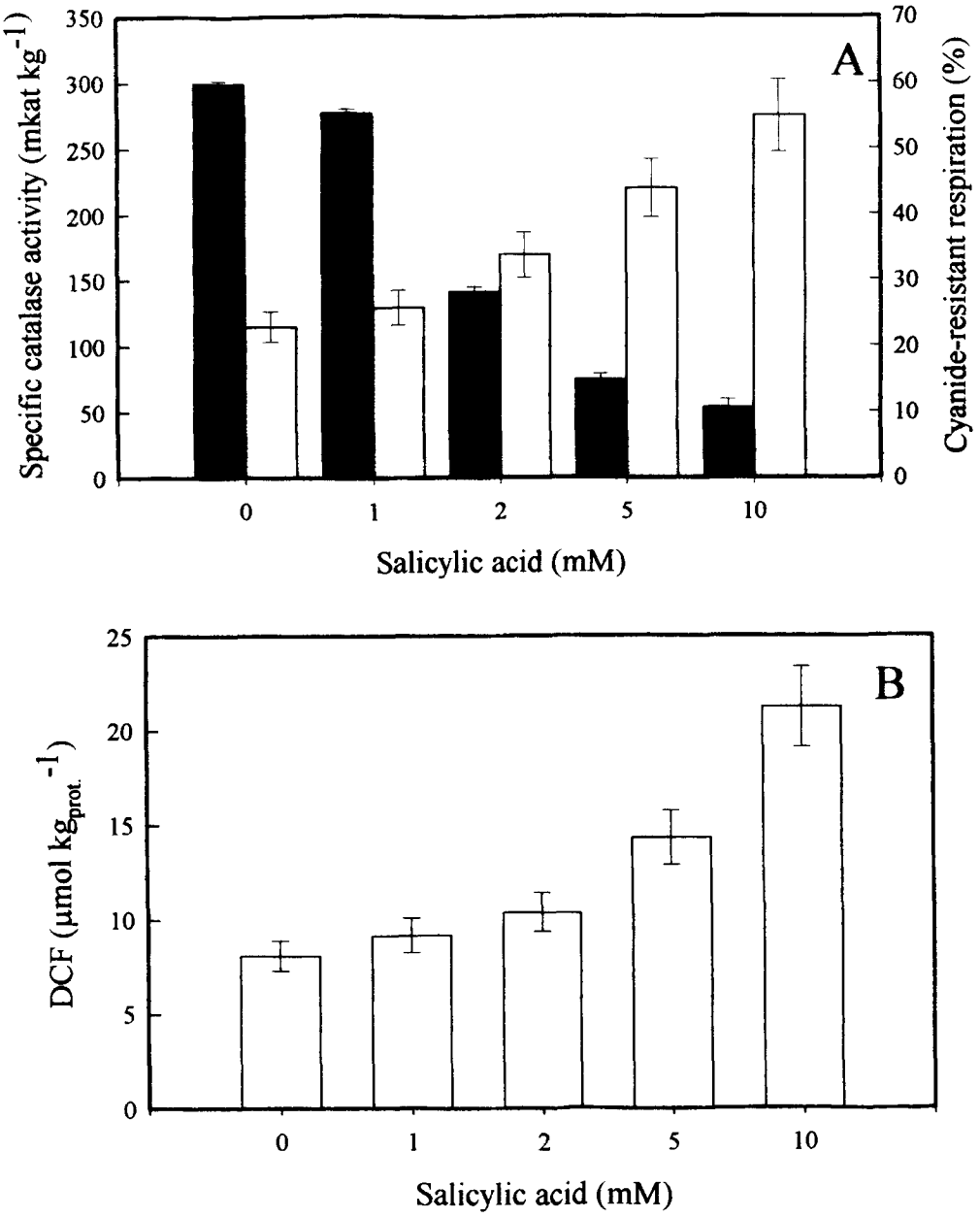

FIGURE 4 Effects of salicylic acid on *A. chrysogenum* cultures. **Part A**. Specific intracellular catalase activities (■) and cyanide-resistant respiration (□). **Part B**. Intracellular peroxide levels

pyruvate[38]), and also on the ratio of reduced ubiquinone to the total ubiquinone pool.[39] A conserved cysteine residue near the N-terminus of the protein is responsible for both disulphide bond formation and organic acid activation of the alternative oxidase.[40] Replacing this cysteine with a serine residue results in a permanently monomeric enzyme that is specifically stimu-

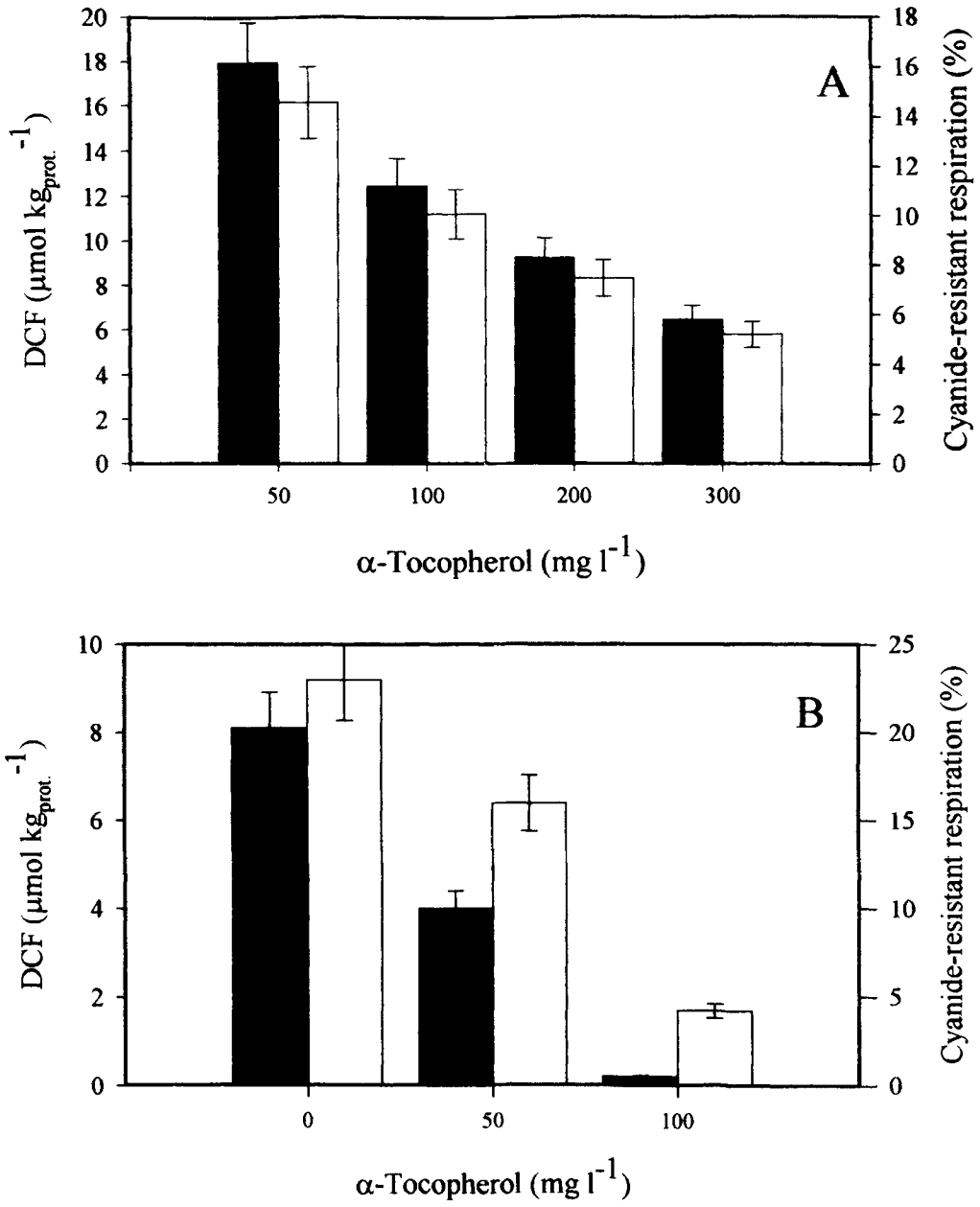

FIGURE 5 Effects of DL-α-tocopherol on *A. chrysogenum* cultures. **Part A**. Cyanide-resistant respiration (□) and intracellular peroxide levels (■) of cultures treated with 200 mM H_2O_2. **Part B**. Cyanide-resistant respiration (□) and intracellular peroxide levels (■) of control cultures

lated by succinate at concentrations of 1–5 mM.[40] These concentrations are likely to be physiologically relevant, as succinate is produced within the mitochondrial matrix where it may become concentrated.

It was just recently established, that in contrast to plant enzymes, alternative oxidases in *Pichia stipitis* and *Neurospora crassa* are permanently monomeric and are not activated directly by α-keto acids.[13] It was also revealed, that a domain of about 40 amino acids responsible for the establishment of the disulphide bond in the plant enzyme is missing in these sequences.[13] Importantly, we found *A. chrysogenum* alternative respiration to be activated by succinate *in vivo*[22], which suggests similar regulatory features to those of *P. stipitis* and *N. crassa*.

In recent years, more and more results suggested that this noncoupled respiration may contribute to the prevention of the generation of reactive oxygen species.[8,9] These are formed under conditions when the cytochrome path is impaired, and can initiate lipid peroxidation and a subsequent damage in membrane structures. In plants, when substrate supply and hence the reduced ubiquinone-pool is too large in the cytochrome pathway, organic acids, especially pyruvate, accumulate and lower the K_m value of the oxidase for ubiquinol in a classical feed-forward regulation.[41] Should the capacity of the alternative route not be sufficient, active oxygen species themselves induce the expression of the alternative oxidase protein, similarly to the H_2O_2-mediated expression of the plant pathogenesis-related proteins.[42] In the light of the recently proposed nature of the fungal alternative oxidase, however, reactive oxygen species might be the only signal towards activation.

When the activity of the enzyme catalase was hindered by salicylic acid[42,43] – the first such observation within the fungal kingdom – intracellular H_2O_2 level increased (Figure 4). This effect was similar to that of the constricted cytochrome path or of the over-reduced ubiquinone-pool, and also results in the stimulation of the alternative pathway. Finally, the effect of the lipid peroxyl radical scavenger DL-α-tocopherol[44] also underlined the solid connection between intracellular peroxide levels and the intensity of the alternative pathway (Figure 5).

Results also shed some light on the underlying processes that take place upon addition of H_2O_2. Since catalase activity is high, H_2O_2 is effectively eliminated, as displayed in Figure 3. The prolonged increase in intracellular peroxide levels thus can only be explained by a secondary, tertiary, etc. production of peroxides due to the propagation of lipid peroxidation by peroxyl radicals.[45] Elimination of these molecules presumably require protective mechanisms other than catalase *in vivo*. Indeed, in *P. chrysogenum* lipid peroxides were found to be eliminated by a glutathione-dependent mechanism.[46]

In fact, the intracellular accumulation of peroxides was inhibited effectively by the addition of vitamin E (Figure 5), which breaks the lipid peroxidation chain reactions by trapping lipid peroxyl radicals.[44,45] Although the intracellular concentration of these molecules is relatively small under physiological conditions, they are very toxic to cells.[46] We found that t-butyl-hydroperoxide was lethal to *A. chrysogenum* mycelia in a concentration of 2 mM (data not shown), which is two orders of magnitudes lower than the highest H_2O_2 concentration used in these experiments.

To sum up, it was established that, similar to higher plants, increased intracellular peroxide levels had a dose-dependent stimulating effect on fungal alternative respiration, while decreased intracellular peroxide concentrations resulted in a dose-dependent decline of its intensity (Figures 1, 4, 5). These findings profoundly strengthen our view on the probable regulation of alternative oxidase by endogenous peroxides in fungi as well. However, further investigations are definitely needed to elucidate the molecular mechanism of the regulation of alternative oxidase gene expression by oxidative stress.[48]

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