Respiratory Characteristics in *Pyricularia oryzae* Exposed to a Novel Alkoxyiminoacetamide Fungicide

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ABSTRACT

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(*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126) inhibited respiration of *Pyricularia oryzae* mycelia by blockade of the electron flux through the cytochrome bc_1 segment of the mitochondrial respiratory chain. However, the mycelia again began to respire at 20 min after treatment. The recovered respiration was sensitive to salicylhydroxamic acid, a potent inhibitor of cyanide-resistant respiration, but not to potassium cyanide, indicating that SSF-126 had the ability to induce cyanide-resistant respiration in *P. oryzae*. The recovered respiration was partially linked to energy transduction because SSF-126 could not completely suppress the mycelial growth. SSF-126 caused a rapid depletion of ATP content, but the ATP levels showed recovery, resulting in improvement of energy charge values. However, the total adenylate pool gradually decreased, resulting in only 60% remaining 48 h after treatment. SSF-126 markedly suppressed the mycelial growth, the duration for mass doubling in cultures incubated with SSF-126 being 2.4 times that in the control ones. Thus, SSF-126 works deleteriously on the supply of metabolic energy in *P. oryzae*.

Additional keywords: adenine nucleotide, complex III, mitochondria, oxidative phosphorylation, submitochondrial particles.

Since Becker et al (4) reviewed the fungicidal activity of strobilurins and other natural compounds, structural modification of these lead compounds has been pursued aggressively. β-Methoxyacrylate and α-methoxyiminoacetates have been introduced as promising candidates for a new class of broad-spectrum fungicides (1,3). We synthesized a number of substituted phenoxyphenyl alkoxyiminoacetamides and evaluated their fungicidal activities against economically important plant diseases. Among the compounds of the series, (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126) was selected as a new candidate fungicide for rice diseases because of its high activity and safety to mammals, fish, and plants.

In eucaryotic microorganisms, electrons generally flow through a cytochrome-mediated pathway in mitochondrial respiratory chains (9,11,25). Energy is conserved in the form of an electrochemical gradient across the mitochondrial inner membrane. This gradient is utilized as the driving force for membrane-associated energy-dependent processes such as ATP synthesis.

In some microorganisms, the mitochondrial respiratory chain contains an alternative (cyanide-resistant) pathway that branches from the normal cytochrome pathway at the level of ubiquinone and terminates with an alternative oxidase. This alternative pathway can be rapidly induced by respiratory inhibitors such as antimycin A and potassium cyanide (KCN) (12–14,19). Such expression of an alternative pathway was found in mycelial cells of *Pyricularia oryzae* Cav. (anamorph of *Magnaporthe grisea,* race 003) exposed to SSF-126. We report on the respiratory characteristics and energy conservation of *P. oryzae* exposed to SSF-126.

MATERIALS AND METHODS

Chemicals. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): antimycin A, cytochrome c (horse heart), defatted bovine serum albumin (BSA), AMP, ADP, ATP. All other reagents were analytical grade and purchased from Wako Chemical Co. (Kyoto, Japan). SSF-126 (99% purity) was synthesized at Aburahi Laboratories, Shionogi Co., Ltd. (Shiga, Japan). KCN was dissolved in distilled water; SSF-126, rotenone, antimycin A in ethanol, and salicylhydroxamic acid (SHAM) in dimethyl sulfoxide.

Fungus and culture conditions. *Pyricularia oryzae* was grown on a culture medium containing 5% (w/w) oatmeal and 2% (w/w) sucrose under black-light blue lamp (FL20S BLB, wavelength scale 352–440 nm, 20W, Toshiba Co., Tokyo, Japan) at 25 C for 6 days. Conidia obtained were transferred to a 5-L flask containing 2 L of potato-dextrose liquid medium (Nissui Co., Tokyo, Japan) at a concentration of 1×10^5 conidia per milliliter and grown aerobically in an orbital shaker (OSI-502, Tokyo Rika Co., Tokyo, Japan) for 24 h at 25 C and 125 rpm.

Preparation of mitochondria and submitochondrial particles. About 20 g (fresh weight) mycelial cells from a 1-day culture of *P. oryzae* were harvested on filter paper (No. 2, Toyo Co., Tokyo, Japan) under vacuum and washed three times with 0.6 M KCl. From the harvested cells, protoplasts of *P. oryzae* were prepared according to the method of Teraoka et al (22). The mitochondria were isolated by suspending the protoplasts in 30 ml of the isolation medium consisting of 250 mM sucrose, 10 mM KCl, 5 mM EDTA, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–Tris buffer (pH 7.2) and 0.15% (w/v) BSA, and sonicating this suspension for 2 s at 0 C. The sonicated suspension was centrifuged at 1,500 g for 10 min, and the supernatant was retained. The precipitate obtained was resuspended in the isolation medium using a glass homogenizer with a loose fitting pestle and centrifuged at 1,500 g for 10 min. The supernatant was combined and recentrifuged at 10,000 g for 20 min at 4 C. The resulting precipitate was washed with the isolation medium without BSA. The mitochondrial fraction was finally suspended in a small volume of the isolation medium without BSA (20–30 mg protein per milliliter) and used as a mitochondrial sample.

To prepare submitochondrial particles, the mitochondria obtained were sonicated for 30 s at 0 C with a sonicator (Cell Disruptor 250, Branson Ultrasonics Co., CT) and centrifuged at 10,000 g for 20 min at 4 C to remove large particles. The supernatant was recentrifuged at 105,000 g for 1 h at 4 C, and the precipitate was washed once with 20 mM HEPES-Tris buffer (pH 7.2). Finally, the resulting precipitate was resuspended in a small volume of the above buffer to a concentration of 10 mg protein per milliliter. Protein was determined by the method of Bradford (6), using BSA as a standard.

Spectral measurement. Inhibition of mitochondrial electron transport was evaluated spectroscopically by measuring the redox state of cytochromes with a Hitachi 557 spectrophotometer (Hitachi Co., Tokyo, Japan) in the split-beam scanning mode with the wavelength scale at 530–630 nm. Submitochondrial particles suspended in 3 ml of air-saturated 20 mM HEPES-Tris buffer (pH 7.2) to a concentration of 2.0 mg protein per milliliter were placed in sample and reference cuvettes. After the baseline had been recorded, SSF-126 (10 μg/mg protein) was added to the sample cuvette and ethanol was added to the reference cuvette. After incubation for 1 min, 5 mM succinate was added to the sample cuvette to reduce the cytochromes. When the reduction was at its maximum, the difference spectrum of succinate-reduced minus air-oxidized cytochromes was recorded at a bandwidth of 1 nm. Complete reduction of cytochromes was achieved with a few crystals of sodium dithionite in the sample cuvette.

Effect of respiratory inhibitors. The rate of oxygen uptake was measured polarographically at 25 C in a sealed Perspex vessel with a circulating water bath, using a Clark-type oxygen electrode (YSI-5300, Yellow Spring Instrument Co., OH). *Pyricularia oryzae* mycelial cells (20 mg fresh weight) from 1-day cultures or submitochondrial particles (2.4 mg protein) were suspended in 3 ml of 20 mM HEPES-Tris buffer (pH 7.2) saturated with air. Inhibitors were added to the suspensions of mycelial cells or submitochondrial particles in appropriate concentrations. Samples treated only with solvent served as controls. The rate of oxygen consumption was calculated from derivative recordings of oxygen electrode signals 2 min after addition of effectors. EC_{50} values were determined from log probability plots.

Induction of cyanide-resistant respiration. Mycelial cells of *P. oryzae* were harvested from 1-day cultures under vacuum, washed with distilled water, and suspended in 20 mM HEPES-Tris buffer (pH 7.0) containing 0.1 M glucose to a mycelial concentration of 6.7 mg (fresh weight)/ml. The mycelial suspensions were treated with 10 μM SSF-126 or antimycin A and incubated at 26 C. At 0, 10, 20, 30, 40, 50, 60, and 120 min after treatment, 3 ml of the mycelial suspension was retrieved from the cultures and transferred into a Perspex vessel. Immediately after, 1 mM KCN was added, and the mixture was incubated for 30 s, followed by addition of 1 mM SHAM. At 90 s later, the rate of oxygen consumption sensitive to SHAM was calculated from derivative recordings of oxygen electrode signals and expressed as nmol O_2 consumption/min/20 mg (fresh weight) mycelia. Cultures receiving only ethanol served as controls. Cyanide-resistant respiration was determined as that sensitive to SHAM in the presence of KCN.

Measurements of ADP/O values and ATP production rate. Cultures grown for 24 h as described above were treated with 10 μM SSF-126 dissolved in ethanol or the solvent alone and incubated for 1 h. Mitochondria were prepared from the cultures as described above. Mitochondria (1.0 mg protein) were suspended in 3 ml of air-saturated respiration medium consisting of 250 mM sucrose, 10 mM KCl, 8 mM KH_2PO_4, 5 mM $MgCl_2$, and 20 mM HEPES-Tris buffer (pH 7.2). ADP/O values were determined from oxygen electrode traces obtained upon addition of 30 μM ADP, according to the method of Chance and Williams (8). The ATP production rate was estimated from the oxygen consumption associated with malate oxidation accelerated by addition of 90 μM or 300 μM ADP, using the following equation: ATP production rate = maximum rate of oxygen consumption $\times$ ADP/O value for malate / intactness of mitochondria. The production rate was expressed as nmol ATP / min / mg protein.

Preparation of cell extracts and HPLC separations. Adenine nucleotides were extracted from *P. oryzae* mycelial cells according to the method of Nakashima (15) with minor modification. Cultures grown for 24 h as described above were treated with SSF-126 (10 μM), SHAM (1 mM), or both. Cultures receiving only solvents served as the control. At 0.2, 0.5, 1, 5, 24, and 48 h after treatment, 1 gram of the cells (fresh weight) was harvested from each culture, suspended in 2 ml of ice-cold 6 % $HClO_4$ and homogenized with a glass homogenizer at 0 C. The homogenates were incubated on ice for 2 h and centrifuged at 2,000 g for 5 min. The precipitate was resuspended in 1 ml of 6% $HClO_4$ and recentrifuged as above. The supernatant was combined and neutralized with 1 N KOH, using a pH meter equipped with a semiconductor sensor, after addition of glycylglycine to a final concentration of 10 mM. The neutralized solution was filtered through a Millipore filter (0.22 μm). The adenine nucleotides were quantified by reversed-phase high-pressure liquid chromatography (HPLC) according to Taylor et al (21) with 20 μl of cell extract being injected onto an HPLC column (Nucleosil 5C18, 4.8 x 300 mm, Chemco Co., Tokyo, Japan) equilibrated with 0.2 N $NH_4H_2PO_4$ (pH 4.1). The column was eluted at 1 ml/min (1,000 p.s.i.), and adenine nucleotides were detected with a UV detector at 254 nm.

Growth of mycelium. Conidia were suspended in 300 ml of potato-dextrose liquid medium culture at a concentration of 1×10^4 conidia per milliliter and preincubated for 24 h at 26 C and 125 rpm. The cultures were then treated with 5 μM or 50 μM SSF-126. Cultures receiving only ethanol served as the control. At 3, 6, 12, and 24 h after treatment, 50 ml of mycelial suspension was retrieved from the cultures and washed with distilled water under vacuum. Dry weights were determined after drying mycelial mats at 80 C for 6 h.

RESULTS

Inhibitory effect on oxygen consumption. SSF-126 dose-dependently inhibited the oxygen consumption by mycelial cells of *P. oryzae*; oxygen consumption was completely inhibited at 10 μM (Fig. 1). SSF-126 also showed an inhibitory effect on the oxygen consumption by submitochondrial particles from the mycelial cells when NADH was used as substrate (Table 1), as did rotenone, antimycin A, and KCN. These respiratory inhibitors occupy different target sites of the mitochondrial respiratory chain. Rotenone inhibits NADH-coenzyme Q oxidoreductase (complex I), antimycin A inhibits ubiquinol-cytochrome-c oxidoreductase (complex III), and KCN inhibits cytochrome-c oxidase (complex IV) (20). Thus, the three phosphorylation sites were operational in the mitochondrial respiratory chain in *P. oryzae*.

Spectral measurements. The difference spectrum (reduced minus oxidized) of submitochondrial particles from mycelial cells, recorded after complete reduction of the cytochromes with sodium dithionite, is shown in Fig. 2. Peaks in the α-region were observed at 550 nm corresponding to cytochromes c + c_1, a broad peak at 562–564 nm representing b, and one at 606–608 nm corresponding to reduced a + a_3. The difference spectrum of submitochondrial particles, reduced with succinate in the presence of SSF-126, showed only one peak in the α-region representing cytochrome b, indicating that cytochromes a + a_3 and c + c_1 were reoxidized, but cytochrome b remained reduced (Fig. 2). The results suggested that SSF-126 interacted with the cytochrome bc_1 segment of the respiratory chain.

Mycelial growth and induction of cyanide-resistant respiration. Mycelial growth of *P. oryzae* was suppressed by addition of SSF-126 (Fig. 3). However, the cultures continued to grow even in the presence of 50 μM SSF-126, which was sufficient to fully

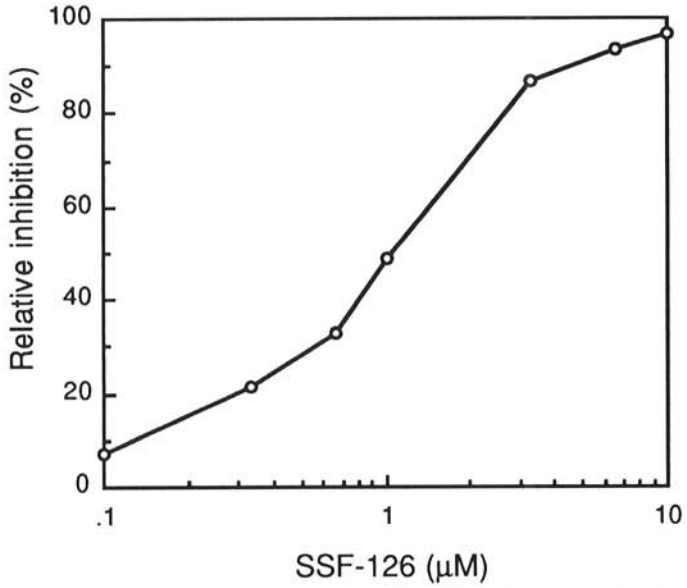

Fig. 1. Effect of (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126) on oxygen consumption by *Pyricularia oryzae* mycelia. Mycelial cells, 20 mg, suspended in 3 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–Tris buffer (pH 7.0) saturated with air; oxygen consumption measured at 25 C. Control activity was 43.2 nmol O_2/min/20 mg f. w. mycelia. Values are means from three experiments. Standard deviations are within 5%.

TABLE 1. Effect of respiratory inhibitors on NADH-oxidation by *Pyricularia oryzae* submitochondrial particles[x]

Inhibitor	Concentration giving half maximal inhibition (μM)[y]
SSF-126[z]	0.24 ± 0.08
Rotenone	0.69 ± 0.06
Antimycin A	0.014 ± 0.001
Potassium cyanide (KCN)	2.8 ± 0.7

[x]Submitochondrial particles (2.4 mg protein) were suspended in 3 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–Tris buffer (pH 7.2). NADH, 1 mM, was added to the suspension, and O_2 consumption was measured polarographically at 25 C. Control activity was 138.2 n mol O_2/min/mg protein.
[y]Values are means ± SD from three experiments.
[z](*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide.

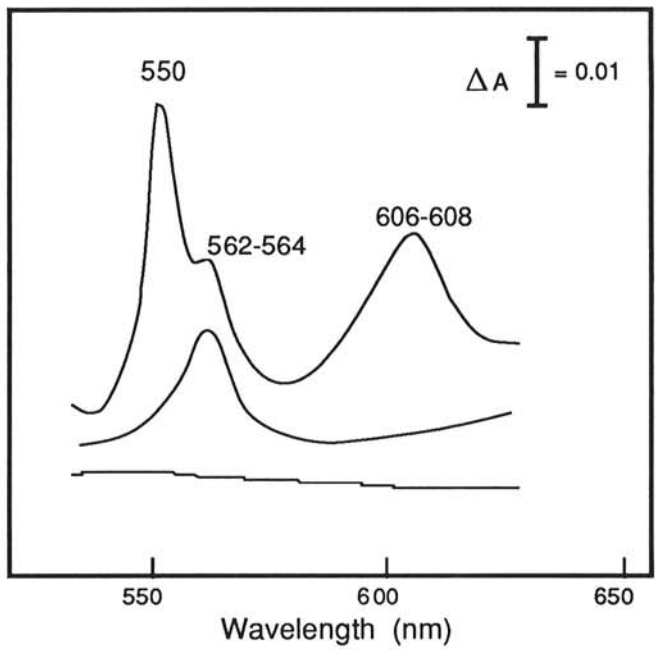

Fig. 2. Difference absorbance spectra of *Pyricularia oryzae* submitochondrial particles in succinate-reduced aerobic state blocked by (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126) and in dithionite-reduced anaerobic state. Spectrum reduced after addition of sodium dithionite (top trace); spectrum reduced after addition of succinate in presence of SSF-126 (middle trace); baseline in bottom trace.

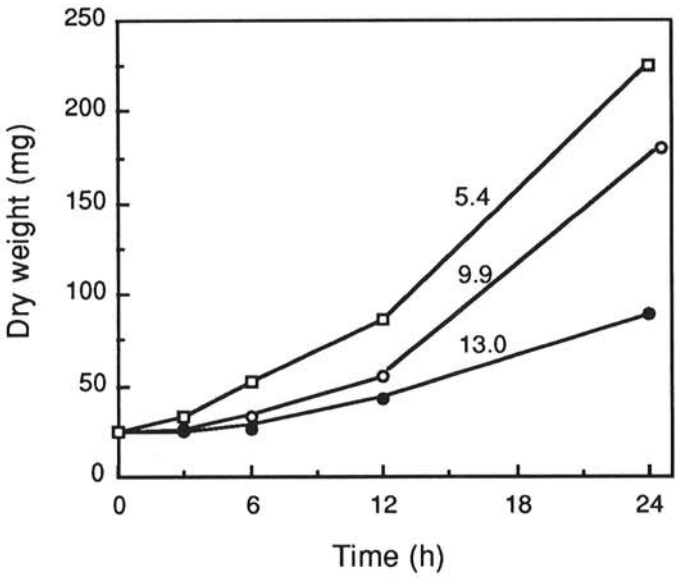

Fig. 3. Growth of *Pyricularia oryzae* cultures after treatment with (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126). Numbers along growth curves are mass doubling times in hours. Treatment with SSF-126 started at time zero. Values are means from two experiments. Standard deviations are within 5%. ○ 5 μM SSF-126, ● 50μM SSF-126, □ control.

inhibit oxygen consumption by mycelial cells when measured immediately after inhibitor treatment (Fig 1.).

Fig. 4 shows the recovery of respiratory activity in mycelial cells treated with SSF-126. At 20 min after treatment with SSF-126, the mycelial cells began to respire again with the respiratory activity rapidly increasing at 30 min after treatment. The rate of respiration reached maximum 60 min after treatment and remained constant thereafter. The recovered respiration was sensitive to SHAM, a potent inhibitor of the cyanide-resistant pathway, but not to KCN, an inhibitor of cytochrome-c oxidase. Similar expression of cyanide-resistant respiration was observed in the treatment with antimycin A (Fig. 4).

Characteristics of mitochondria and adenylate contents in *P. oryzae*. The percent intactness of isolated mitochondria ranged from 66 to 85% with a mean value of 76% (data not shown) when evaluated by the method described by Neuberger et al (16). Control mitochondria gave a ADP/O value of 2.19 for 5 mM malate, 1.43 for 15 mM succinate and 0.69 for 5 mM ascorbate coupled with 1 mM tetramethyl-1, 4-phenylene diamine.

With mitochondria from mycelial cells treated with SSF-126, the ADP/O value for malate was very low (0.79 instead of 2.19 in control) and was unaffected by addition of KCN (Table 2). Furthermore, the mitochondria gave the ADP/O value of 0 in both malate oxidation in the presence of SHAM and succinate oxidation.

With control mitochondria, the oxidative phosphorylation was accelerated by ADP added externally, in particular, being enhanced more markedly at a higher concentration of ADP (Table 3). By contrast, with mitochondria from cells treated with 10 μM SSF-126, the phosphorylation was not enhanced as much by the increase of external ADP concentration as that observed in control mitochondria.

Mycelial cells treated with SSF-126, SHAM, or both were harvested at various times and assayed for adenine nucleotides (Fig. 5). In control cells, the main contribution to the adenylate pool was the ATP content, and AMP levels were generally low while ADP contributed to the adenylate pool. A similar tendency was observed in the cells treated with SHAM. The energy charge (EC = [ATP]+1/2[ADP]/[ATP]+[ADP]+[AMP]), calculated according to Atkinson (2), ranged from 0.78 to 0.87 for control cells and 0.82 to 0.89 for the cells treated with SHAM (Table 4).

In the cells treated with SSF-126, the ATP content was rapidly depleted by 0.2 h after treatment and was replaced in the adenylate pool by AMP, leading to a marked decrease of the energy charge (EC = 0.40). However, the ATP levels of the cells gradually rose thereafter, and the content amounted to about 75% of the control at 48 h after treatment. The EC values of the cells began to rise 0.5 h after treatment, improving to control levels at 24 h after treatment. However, the total adenylate pool of the cells

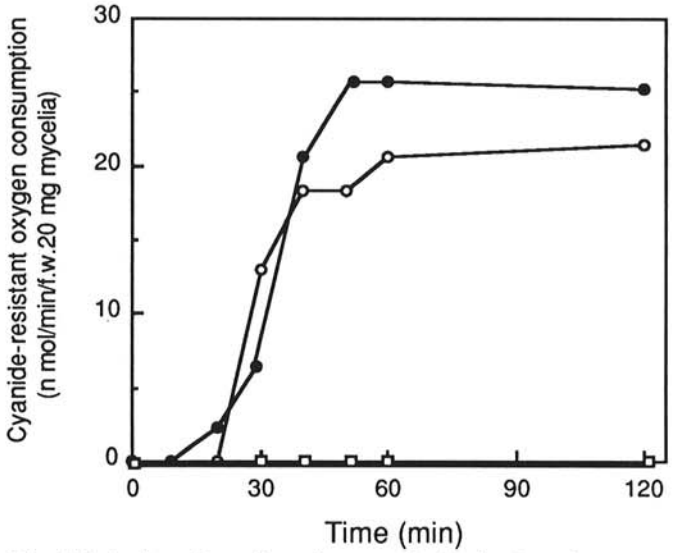

Fig. 4. Induction of cyanide-resistant respiration in *Pyricularia oryzae* with (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126). Cyanide-resistant respiration was measured as oxygen consumption sensitive to salicylhydroxamic acid in the presence of 1 mM potassium cyanide. Values are the means from three experiments. Standard deviations are within 5%. ○ 10 μM SSF-126, ● 10 μM antimycin A, □ control.

TABLE 2. Effect of respiratory inhibitors on ADP/O values of *Pyricularia oryzae* mitochondria treated with (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126)[z]

Inhibitor	ADP/O	
	Succinate	Malate
None	0	0.79 ± 0.04
Potassium cyanide (KCN)	0	0.76 ± 0.02
Salicylhydroxamic acid (SHAM)	0	0

[z] Mitochondria were prepared from *P. oryzae* mycelia after growth for 24 h and additional incubation for 1 h with 10 μM SSF-126. Mitochondria (1 mg protein) were suspended in 3 ml of a respiration medium containing 10 μM SSF-126, and oxygen consumption was measured at 25 C. Succinate, 15 mM; ADP, 30 μM; KCN, 1 mM; SHAM, 1 mM.

TABLE 3. Rate of ATP production in *Pyricularia oryzae* mitochondria treated with (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126)

ADP (n mol/ml)	ATP production rate[x] (n mol ATP/min/mg protein)	
	Treated mitochondria[y]	Control mitochondria
90	44.4 a ± 1.8[z]	125.9 b ± 17.5
300	67.6 a ± 7.1	613.2 b ± 64.7

[x] Estimated from O_2 consumption accelerated by ADP addition.
[y] Mitochondria were prepared from *P. oryzae* mycelia after growth for 24 h and additional incubation for 1 h with 10 μM SSF-126. Malate, 5 mM, was used as the respiratory substrate.
[z] Values are the means ± SD from three experiments. Values followed by different letter (within row) differ significantly ($P < 0.025$).

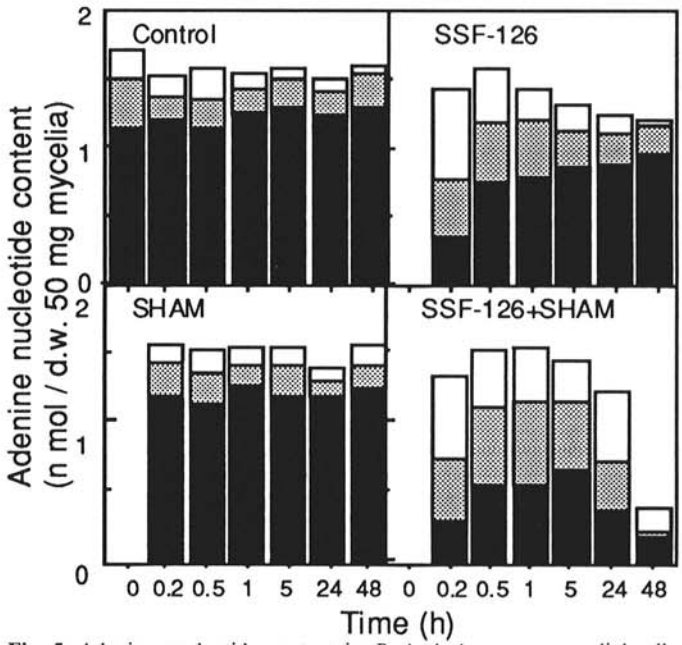

Fig. 5. Adenine nucleotide content in *Pyricularia oryzae* mycelial cells treated with respiratory inhibitors. Treatments with (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide (SSF-126), salicylhydroxamic acid, or both, started at time zero. Values are means from three experiments (experimental variation between 0.02 and 0.2 nmol/50 mg d.w. for ATP and sum; between 0.01 and 0.05 nmol/50 mg d.w. for ADP and AMP). ■ ATP, ■ ADP, □ AMP.

decreased gradually, amounting to only 60% of the control at 48 h after treatment (Fig. 5).

When SSF-126 was combined with SHAM, each adenylate contributed almost equally to the adenylate pool of the cells, and the ATP content remained at lower levels over the experimental period. The combined effect on EC values was strong, with no recovery observed (Table 4). In particular, both the ATP levels and the total adenylate pool were markedly limited in the treated cells at 48 h.

DISCUSSION

Spectral measurements showed that SSF-126 inhibited respiration of *P. oryzae* by blockade of the electron flux through the cytochrome bc_1 segment in the mitochondrial respiratory chain (Fig. 6), an effect similar to that of other compounds containing the (*E*)-β-methoxyacrylate group such as myxothiazol, oudemansin A, and strobilurins (4,7,23,24). After treatment with SSF-126, respiratory activity recovered with time and was sensitive to SHAM but not to KCN. Thus, SSF-126 induced cyanide-resistant respiration in mycelial cells of *P. oryzae*. Similar expression of cyanide-resistant respiration was also observed in *Neurospora crassa* and *Hansenula anomala* incubated with antimycin A or KCN. Such response to respiratory inhibitors may be a general characteristic in microorganisms.

Mitochondria from the mycelial cells treated with SSF-126 gave ADP/O values close to 1 for malate oxidation and 0 for succinate oxidation. This indicates that the electrons must diverge to the alternative pathway before they enter complex III, and that no phosphorylation site is associated with the cyanide-resistant pathway. Thus, SSF-126 causes a marked decrease in the efficiency of oxidative phosphorylation by by-passing phosphorylation sites 2 and 3 (indicated in Fig. 6) and feeding electrons to the alternative pathway.

The rate of ATP production was markedly depressed when the electrons were diverted to the alternative pathway upon oxidation of the NAD-linked substrate. The decrease of production rate was primarily due to the fact that only site 1 (indicated in Fig. 6) was operational for phosphorylation in the mitochondria. Furthermore, the production rate was almost independent of the increasing external ADP concentration in the mitochondria though it markedly increased in control ones. This suggests that the electron flux is limited to the depressed rate in the mitochondria by the alternative oxidase. The rate of enzyme reaction is mainly dependent on the affinity to a substrate, concentration of substrate or enzyme, and molecular activity of enzyme. The affinity of the alternative oxidase for molecular oxygen is lower (1–2 μM) than that of cytochrome oxidase (0.1–0.6 μM) (5). However, at the molecular oxygen concentration (240 μM) utilized in this study, the lower affinity for molecular oxygen is not primarily responsible for the marked decrease of the phosphorylation rate in the mitochondria. It seems that the electron flux through the alternative pathway is depressed by low molecular activity or the limited quantity of the oxidase. The rate of ATP production in the mitochondria from mycelial cells treated with SSF-126 is probably suppressed by the low phosphorylation efficiency and the characteristics of the oxidase.

The inhibition of the cytochrome pathway by SSF-126 caused a rapid decrease in the ATP content of mycelial cells, which in turn accumulated a high amount of AMP, resulting in a marked decrease in the EC value. However, the ATP content recovered gradually with time, accompanied by improvement in the EC values, which was probably due to ATP production at complex I. Although the EC values showed a tendency toward recovery, SSF-126 caused an irreversible decrease in total adenylate pool levels; this may lead to altered enzyme activity even with improvement of the EC value (18).

When SSF-126 was combined with SHAM, a potent inhibitor of alternative oxidase, the ATP content of mycelial cells decreased markedly immediately after treatment. However, a transient elevation of ATP levels was observed in the mycelial cells for up to 5 h after treatment. This partial recovery of the ATP content is probably due to ATP derived from glycolysis because electron flux through both the cytochrome and alternative pathways is completely inhibited under such conditions. With a longer term of treatment, the combined effect on both the total adenylate pool and EC values was strong, resulting in depletion of the ATP content and restraint of mycelial growth.

TABLE 4. Effect of SSF-126[x] and SHAM[y] on adenylate energy charge of *Pyricularia oryzae* mycelia[z]

Time (h)	Energy charge			
	Control	SSF-126	SHAM	SSF-126 + SHAM
0	0.78 ± 0.04			
0.2	0.85 ± 0.01	0.40 ± 0.11	0.84 ± 0.01	0.38 ± 0.06
0.5	0.80 ± 0.05	0.60 ± 0.05	0.82 ± 0.03	0.54 ± 0.09
1	0.85 ± 0.03	0.71 ± 0.08	0.86 ± 0.08	0.58 ± 0.05
5	0.86 ± 0.01	0.76 ± 0.04	0.84 ± 0.05	0.63 ± 0.02
24	0.84 ± 0.01	0.81 ± 0.04	0.89 ± 0.08	0.44 ± 0.02
48	0.87 ± 0.03	0.83 ± 0.07	0.85 ± 0.04	0.49 ± 0.06

[x](*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide.
[y]Salicylhydroxamic acid.
[z]Treatments with SSF-126, SHAM, or both started at time zero. Values are the means ± SD from three experiments. SSF-126, 10 μM; SHAM 1 mM.

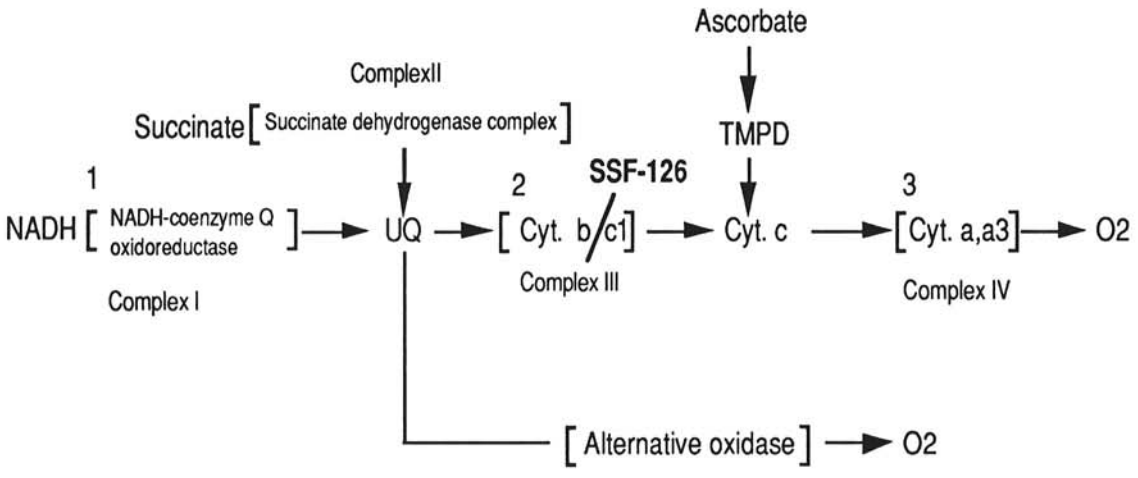

Fig. 6. Schematic diagram of electron transport pathway and inhibition site of (*E*)-2-Methoxyimino-N-methyl-2-(2-phenoxyphenyl) acetamide in the mitochondrial respiratory chain in *Pyricularia oryzae*. Coupling sites of electron flow to proton gradient formation are shown by numbers 1, 2, and 3. UQ, ubiquinone; cyt., cytochrome.

Respiratory inhibitors have fungistatic rather than fungicidal effects against *Ustilago nuda;* the initial fungistatic effects becoming fungicidal through autolysis (17). Carboxin, a systemic fungicide that inhibits electron flux through the mitochondrial succinate dehydrogenase complex (complex II) (10,26), shows a fungistatic effect on *U. nuda.* This fungicide can decrease energy production in the fungus by the electron flux inhibition, but other pathways linked to energy conservation, such as oxidation of NAD-linked substrates and of cytosolic NADH through the mitochondrial respiratory chain, probably remain functional. Antimycin A also shows fungistatic effects (17) and may depress energy production in a fashion similar to SSF-126, because both agents inhibit electron flux through complex III and induce the alternative pathway (12,13). This is a probable reason for fungistatic rather than fungicidal effects of respiratory inhibitors.

Finally, our results show that SSF-126 inhibits the electron flux through the cytochrome bc_1 segment in the mitochondrial respiratory chain and causes a marked decrease in the efficiency of oxidative phosphorylation by diverting electrons to the alternative pathway. When this occurs, the rate of ATP production is markedly depressed in the mitochondria and the mycelial growth is suppressed. Thus, SSF-126 works deleteriously on the supply of metabolic energy in *P. oryzae*.

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