Original Article

Cyanide-insensitive Respiration of Phytopathogenic Fungi Demonstrated by Antifungal Joint Action of Respiration Inhibitors

Keisuke HAYASHI, Masanori WATANABE, Toshinobu TANAKA and Yasuhiko UESUGI

Ube Research Laboratory, Ube Industries, Ltd., Tokiwadai, Ube 755, Japan

(Received November 6, 1995; Accepted July 19, 1996)

A survey was conducted for involvement of cyanide-insensitive respiration of phytopathogenic fungi by testing a joint fungitoxic action between salicylhydroxamic acid (SHAM) as an inhibitor of the cyanide-insensitive pathway and the inhibitors of cytochrome pathway of respiratory chain such as methoxyacrylate derivatives or antimycin A. Remarkable synergism in fungitoxic action was shown in *Botrytis cinerea*, *Monilinia fructicola* and *Pyricularia oryzae*. The synergism may result from the induction of the alternative pathway by the latter inhibitors and the inhibition of the induced alternative pathway by SHAM. To confirm the induction of the alternative pathway, effects of SHAM on oxygen consumption by hyphal cells of three fungi were tested by pretreating with methoxyacrylate or potassium cyanide. These results confirmed the induction of cyanide-insensitive respiration by the pretreatment with methoxyacrylate or potassium cyanide as expected from the joint fungitoxic action. The synergism in fungitoxic action was also observed in *Cochliobolus miyabeanus*, though it was less remarkable. In *Rhizoctonia solani*, the synergism was observed with methoxyacrylates but hardly with antimycin A, so that cyanide-insensitive respiration was induced with the methoxyacrylates but hardly with antimycin A in *Rhizoctonia solani*. In *Gibberella fujikuroi*, *Pythium* sp. and *Valsa ceratosperma* the synergism was not necessarily clear. A phytopathogenic bacterium, *Xanthomonas campestris* pv. *citri* and a yeast, *Saccharomyces cerevisiae* did not show any synergism in joint antimicrobial action of the above inhibitors.

INTRODUCTION

Cytochrome pathway in respiratory chain is important for aerobic organisms, and the inhibitors of the pathway are fatal to most aerobic organisms. To some organisms, however, the inhibitors are not fatal because an alternative pathway bypassing cytochromes could be formed (Fig. 1). Phytopathogenic fungi, *Ustilago maydis*,^{1,2)} *Ceratocystis ulmi*¹⁾ and *Fusarium lini*³⁾ have already been reported to exhibit such an alternative pathway. Recently a methoxyacrylate analogue SSF-126 was reported to induce a cyanide-insensitive pathway of respiration in *Pyricularia oryzae*.^{4,5)} It was also observed with another derivative of methoxyacrylate in *Botrytis cinerea*.⁶⁾ These results may explain the reason why methoxyacrylate fungicides often do not exhibit fungitoxicity in ordinary *in vitro* tests. At the same time, existence of the alternative pathway might be rather popular in phytopathogenic fungi than it has been reported.

In the present experiment, a survey of the alternative

pathway among phytopathogenic fungi was conducted by two methods. One is the test for joint fungitoxic action between an inhibitor of alternative pathway, salicylhydroxamic acid (SHAM), and inhibitors of cytochrome pathway such as methoxyacrylate derivatives or antimycin A, using crossed paper technique.^{7,8)} The other is the test for oxygen consumption by the fungal cells with and without pretreatment with a methoxyacrylate or with potassium cyanide. The pretreatment was expected to inhibit the cytochrome pathway and induce the alternative pathway. These actions by the pretreatment were checked by the effects of the inhibitors on the oxygen consumption by the fungal cells.

MATERIALS AND METHODS

1. Compounds

A methoxyacrylate, (*E*)-methyl 3-methoxy-2-(*O*-*E*-styrylphenyl)acrylate (**I**), was chosen for the present experiment because it showed a typical joint fungitoxic action with SHAM in the preliminary tests. Another methoxyacrylate analogue, (*E*)-2-methoxyimino-*N*-meth-

Fig. 1 Electron transfer in mitochondrial respiratory chain and the inhibitors.

CoQ: coenzyme Q.

Fig. 2 Chemical structures of tested methoxyacrylate derivatives (I, II) and SHAM (III).

yl-2-(2-phenoxyphenyl)acetamide (SSF-126, II), was also tested. These methoxyacrylate derivatives were synthesized according to the procedure described in the respective patents. Antimycin A (Sigma Chem. Co.), SHAM (Sigma Chem. Co.) and other chemicals were commercially obtained.

2. Test Microbes

Test phytopathogenic fungi and other microbes and their sources are stated in Table 1. They are kept on PDA medium at 25°C.

3. Test for Joint Fungitoxic Action

Crossed paper technique^{7,8)} was used to observe joint fungitoxic action between SHAM and inhibitors of cyto-

chrome pathway such as methoxyacrylate I, its analogue II or antimycin A. Cell suspension of test microbes were obtained from conidia of *P. oryzae* produced on 7 day culture on oatmeal medium, and from conidia of *Alternaria kikuchiana*, *B. cinerea*, *Cochliobolus miyabeanus*, *Gibberella fujikuroi*, *Monilinia fruticola* and *Valsa ceratosperma*, hyphal cells of *Pythium* sp. and *Rhizoctonia solani*, and yeast cells of *Saccharomyces cerevisiae* and bacterial cells of *Xanthomonas campestris* pv. *citri* each produced on 7 day culture on PDA medium. The cell suspensions were added to PDA medium at about 60°C to make test agar plates. Strips of filter paper (7×80 mm) were impregnated with acetone solution of test inhibitors, air-dried and placed on the test agar plates to form a cross inhibition.

4. Test for Oxygen Consumption by Phytopathogenic Fungi

Test fungi, *B. cinerea*, *M. fruticola* and *P. oryzae* were cultured in potato dextrose broth (PDB) for 3 days at 25°C with shaking, and hyphal cells were harvested by filtration through a Buechner funnel and washed twice with distilled water. Test yeast *S. cerevisiae* were also cultured in PDB for 3 days at 25°C with shaking, and yeast cells were collected by centrifugation (3000×g, 10 min) and washed twice with distilled water. The cells (0.25 g fresh weight) were suspended in 5 ml of 50 mM phosphate buffer (pH 6.5), preincubated at 30°C for 15 min and incubated further with or without addition of final 10 μM of methoxyacrylate I or final 1 mM of potassium cyanide for 1 hr, and the suspension was used for the next test.

An aliquot (0.1 ml) of the cell suspension was added to 0.9 ml of 50 mM phosphate buffer (pH 6.5) at 30°C containing 50 mM sucrose in a cell of polarographic apparatus (Oxygraph Type 8 of Central Science Co.). Potassium cyanide (final 0.5 mM) and SHAM (final 2 mM) were added consecutively at a time interval during

Table 1 Test microbes and their sources.

Test microbes	Sources
<i>Alternaria kikuchiana</i>	Tottori University
<i>Botrytis cinerea</i> <i>Cochliobolus miyabeanus</i> <i>Gibberella fujikuroi</i> <i>Pythium</i> sp. <i>Rhizoctonia solani</i> <i>Valsa ceratosperma</i>	The Institute of Physical and Chemical Research, RIKEN
<i>Monilinia fruticola</i>	Nagano Prefectural Agricultural Experiment Station
<i>Pyricularia oryzae</i> <i>Xanthomonas campestris</i> pv. <i>citri</i>	Isolates from the field in Yamaguchi prefecture collected by author's laboratory
<i>Saccharomyces cerevisiae</i>	Tokyo University of Agriculture

the course of oxygen consumption, which was recorded graphically by the apparatus.

RESULTS

1. Joint Action of Respiration Inhibitors

The synergism in fungitoxic action between an inhibitor of alternative pathway, SHAM, and inhibitors of

cytochrome pathway, methoxyacrylate **I**, methoxyacrylate analogue **II** or antimycin A was observed in all the test phytopathogenic fungi, though it was less remarkable in *G. fujikuroi*, *Pythium* sp., *V. ceratosperma*. In *R. solani*, the synergism by SHAM with methoxyacrylates is remarkable but less with antimycin A. In *S. cerevisiae* and *X. campestris* pv. *citri*, there was no synergistic action. The results are summarized in Table 2. The synergism in fungitoxic action between methoxyacrylate **I** and SHAM in *B. cinerea* and *M. fructicola* are shown in Fig. 3.

Fig. 3 Synergism in fungicidal action between methoxyacrylate **I** and SHAM in *Botrytis cinerea* (upper left) and *Monilinia fructicola* (upper right).

The vertical and horizontal paper stripes were impregnated with acetone solution of **I** (500 mg/l) and of SHAM (5g/l) respectively. The antifungal activities of **I** alone (lower left) and SHAM alone (lower right) at the same dosages in *M. fructicola* are also shown for reference.

2. Oxygen Consumption in Phytopathogenic Fungi

Oxygen consumption in *P. oryzae* without preincubation was 0.288 $\mu\text{mol O}_2/\text{min/g}$ wet cell. By adding 0.5 mM KCN (final) this respiration was inhibited completely. Respiration activity in *P. oryzae* preincubated with 10 μM methoxyacrylate **I** was 0.119 $\mu\text{mol O}_2/\text{min/g}$ wet cell. This respiration was inhibited just slightly at 0.5 mM KCN and the further addition of 2 mM SHAM completely stopped the respiration. Oxygen consumption in *P. oryzae* preincubated with 1 mM KCN was 0.169 $\mu\text{mol O}_2/\text{min/g}$ wet cell and the respiration was slightly inhibited at 0.5 mM KCN. This respiration was completely inhibited by adding 2 mM SHAM. Similar results were obtained in the other phytopathogenic fungi, *B. cinerea* and *M. fructicola*. The respiration in *S. cerevisiae* preincubated with 10 μM methoxyacrylate **I** or 1 mM KCN was completely inhibited at 0.5 mM KCN. The data of *B. cinerea*, *M. fructicola*, *P. oryzae* and *S. cerevisiae* are described in Table 3, and the records of oxygen consumption with *B. cinerea* and *S. cerevisiae* are shown in Fig. 4.

Table 2 Synergistic fungitoxic action of an inhibitor of the alternative pathway, SHAM, with inhibitors of cytochrome pathway, methoxyacrylate **I**, **II** or antimycin A.

Test microbes	Inhibitors of cytochrome pathway		
	Methoxyacrylate I	Methoxyacrylate II	Antimycin A
<i>Alternaria kikuchiana</i>	+	±	±
<i>Botrytis cinerea</i>	++	+	++
<i>Cochliobolus miyabeanus</i>	++	±	++
<i>Gibberella fujikuroi</i>	±	±	—
<i>Monilinia fructicola</i>	++	++	++
<i>Pyricularia oryzae</i>	++	+	++
<i>Pythium</i> sp.	±	±	—
<i>Rhizoctonia solani</i>	++	+	—
<i>Valsa ceratosperma</i>	±	—	±
<i>Saccharomyces cerevisiae</i>	—	—	—
<i>Xanthomonas campestris</i> pv. <i>citri</i>	—	—	—

Synergism in fungitoxic action observed by crossed paper technique between SHAM at 5 g/l of acetone solution used for impregnation of paper strips and inhibitors of cytochrome pathway at 20, 100 and 500 mg/l of acetone solution. Level of synergism is expressed by the dosage of inhibitors of cytochrome pathway which exhibit expansion of growth inhibitory zone at the intersection of test paper strips as follows: ++, at 20 mg/l; +, at 100 mg/l; ±, at 500 mg/l; —, synergism was not observed at 500 mg/l of acetone solution.

Table 3 Effects of respiration inhibitors on oxygen consumption in phytopathogenic fungi.

Microbes	Pretreated with	O ₂ consumption ($\mu\text{mol O}_2/\text{min/g wet cell}$)	Inhibition (%) by consecutive addition of	
			0.5 mM KCN	2 mM SHAM
<i>Botrytis cinerea</i>	None	0.397	92.1	—
	10 μM methoxyacrylate I	0.066	4.8	100
	1 mM KCN	0.069	31.8	100
<i>Monilinia fructicola</i>	None	0.056	100	—
	10 μM methoxyacrylate I	0.066	8.6	100
	1 mM KCN	0.047	35.1	100
<i>Pyricularia oryzae</i>	None	0.288	100	—
	10 μM methoxyacrylate I	0.119	21.0	100
	1 mM KCN	0.169	31.5	100
<i>Saccharomyces cerevisiae</i>	None	0.522	100	—
	10 μM methoxyacrylate I	0.047	100	—
	1 mM KCN	0.025	100	—

Fig. 4 The records of time course of oxygen consumption in *Botrytis cinerea* (a), *B. cinerea* pretreated with 10 μM I (b), *B. cinerea* pretreated with 1 mM KCN (c), and *Saccharomyces cerevisiae* (d), *S. cerevisiae* pretreated with 10 μM I (e), *S. cerevisiae* pretreated with 1 mM KCN (f).

Arrows on the graphs indicate the time of addition of inhibitors.

DISCUSSION

A method was proposed to detect cyanide-insensitive pathway in fungal respiration. It is based on the joint fungitoxic action by the combination of an inhibitor of the alternative pathway, SHAM, with the inhibitors of cytochrome pathway. If the alternative pathway is not present in the test fungal cells, no joint action is expected between a set of the inhibitors. When the alternative pathway is formed in the fungal cells, joint inhibition of both the respiratory pathways is necessary for fungitoxicity. Therefore, inhibition of the fungal growth is

exhibited only in the presence of both SHAM and a inhibitor of cytochrome pathway. This joint fungitoxicity is easily assayed by crossed paper technique. Since co-existence of both SHAM and an inhibitor of cytochrome pathway is expected only around the intersection of the two paper strips impregnated with the respective inhibitors, the growth-inhibitory zone must be remarkable only around the intersection.

This method was applied on various phytopathogenic fungi, because phytopathogenic fungi reported to exhibit the alternative respiratory pathway are rather limited. The result clearly suggested its existence in *B. cinerea*, *M.*

fruticola and *P. oryzae*. The other test fungi also exhibited the similar joint fungitoxic action, but the synergism was less remarkable in some test fungi, such as *G. fujikuroi*, *Pythium* sp. and *V. ceratosperma*. In *G. fujikuroi*, *Pythium* sp. and *R. solani*, synergism of SHAM with methoxyacrylates was more or less observed but not with antimycin A. Thus antimycin A might be less active in induction of alternative pathway in these fungi. But none of the test phytopathogenic fungi seemed entirely lacking the alternative pathway. On the other hand, the yeast *S. cerevisiae* and the bacterium *X. campestris* pv. *citri* did not exhibit any synergism in joint antimicrobial activity between SHAM and inhibitors of cytochrome pathway under the condition of the present experiment. These results coincided with the effects of SHAM on the oxygen consumption of microbial cells with and without pretreatment with cytochrome inhibitors.

The induction of an alternative respiratory pathway in fungal cells may decrease the fungitoxicity of inhibitors of cytochrome pathway in respiratory chain. In fact, methoxyacrylate derivatives sometimes lack their fungitoxicity in *in vitro* tests. A methoxyacrylate analogue **II** was reported to be not necessarily a potent fungitoxicant in *in vitro* tests but it is effective to control rice blast because flavonoids in rice plants are inhibitors of the alternative respiratory pathway. And the joint inhibition of the analogue **II** and the flavonoids seemed the reason of the effectiveness.⁴⁾ The test for joint fungitoxic action of SHAM with another compounds may be a hopeful method to find the inhibitors of cytochrome respiratory pathway in phytopathogenic fungi.

In some plants, amounts of flavonoids are not necessarily enough to inhibit the alternative respiratory pathway. In such cases, combination of inhibitors of alternative pathway with methoxyacrylate fungicides might be effective to control plant diseases. Screening of such inhibitors may be conveniently conducted by the crossed paper technique.

Since cyanide-insensitive respiratory pathway was suggested to exist in a wide range of phytopathogenic fungi, it is an unavoidable factor to be investigated in the research on fungal respiration. Test methods using novel respiration inhibitors may be an useful tool in the investigation.

REFERENCES

- 1) J. L. Sherald & H. D. Sisler : *Plant Physiol.* **46**, 180 (1970)
- 2) J. L. Sherald & H. D. Sisler : *Plant Cell Physiol.* **13**, 1039 (1972)
- 3) E. Weiss-berg & C. Tamm : *Experientia* **27**, 778 (1971)
- 4) H. Yukioka, A. Mizutani, H. Tamura, N. Miki, M. Masuko & R. Takeda : Abstr. ; Annu. Meet. Pestic. Sci. Soc. Jpn., p. 100, 1994
- 5) A. Mizutani, H. Yukioka, H. Tamura, N. Miki, M. Masuko & R. Takeda : *Phytopathology* **85**, 306 (1995)
- 6) M. Watanabe, K. Hayashi, T. Tanaka & Y. Uesugi : Proc. ; 11th Int. Symp. Modern Fungicides and Antifungal Compounds, Reinhardtbrunn, Germany, pp. 111-115, 1995
- 7) M. Katagiri & Y. Uesugi : *Phytopathology* **67**, 1415 (1977)
- 8) H. Sugiura, K. Hayashi, T. Tanaka, M. Takenaka & Y. Uesugi : *Pestic. Sci.* **39**, 193 (1993)

要 約

呼吸阻害剤の抗菌連合作用により示された植物病原菌のシアン耐性呼吸

林 敬介, 田中敏房, 渡辺正徳, 上杉康彦

植物病原菌のシアン耐性呼吸の有無を調べる目的で, シアン耐性呼吸の阻害剤であるサリチルヒドロキسام酸 (SHAM) と呼吸鎖シトクローム電子伝達系の阻害剤であるメトキシアクリレート系化合物やアンチマイシン A との抗菌連合作用を調べた。 *Botrytis cinerea*, *Monilinia fruticola* および *Pyricularia oryzae* において顕著な抗菌協力作用が認められた。このことから, シトクローム系阻害剤によるシアン耐性呼吸の誘導と SHAM によるその阻害が考えられた。それを確かめる目的で, シトクローム系阻害剤で処理したこれら 3 病原菌の菌糸体の酸素消費と, その酸素消費に及ぼすシアン耐性呼吸阻害剤の影響を調べた。その結果, これら 3 病原菌におけるシアン耐性呼吸の誘導が確認された。抗菌協力作用は, *Cochliobolus miyabeanus* においても小さいながら認められ, シアン耐性呼吸の存在が示唆された。 *Rhizoctonia solani* では, メトキシアクリレート系化合物では協力作用が認められたが, アンチマイシン A においては認められず, シアン耐性呼吸の誘導が, アンチマイシン A では起こりにくいと考えられた。 *Gibberella fujikuroi*, *Pythium* sp., *Valsa ceratosperma* における抗菌協力作用は, 本実験では顕著ではなかった。病原細菌の *Xanthomonas campestris* pv. *citri* および酵母 *Saccharomyces cerevisiae* においては, 抗菌協力作用はまったく認められなかった。