Original Article

Mode of Action of Copper 8-Quinolinolate on *Alternaria kikuchiana*

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Spore germination, hyphal elongation and hyphal penetration of *Alternaria kikuchiana* were observed on alcohol-treated epidermal strips of onion bulb. I_{50} of copper sulfate, 8-quinolinol and copper 8-quinolinolate on germination of *A. kikuchiana* were 10^{-3} M, 10^{-4} M and 10^{-6} M respectively, and those on penetration were 3×10^{-4} M, 3×10^{-5} M and 10^{-6} M respectively, indicating that copper ion and 8-quinolinol acted synergistically against *A. kikuchiana*. The synergism was more remarkable when 8-quinolinol existed more than twice the concentration of copper ion. On the contrary, the synergism was less remarkable when copper ion existed more than a half the concentration of 8-quinolinol. 8-Quinolinol at 10^{-2} M accelerated state 4 respiration of rat liver mitochondria to 2.5 times and showed uncoupling effect on oxidative phosphorylation at higher than 10^{-3} M. 8-Quinolinol also accelerated respiration of the mitochondrial fraction of *A. kikuchiana* to 1.4 times at 2×10^{-3} M. Thus a possibility was suggested that the inhibitory effect of 8-quinolinol on the growth of *A. kikuchiana* is related to the uncoupling effect. Since mitochondrial membrane is generally permeable to uncouplers, the fungal cell membrane which is similar to mitochondrial membrane by nature may also be permeable to 8-quinolinol and permit copper ion to pass the barrier along with 8-quinolinol as a complex to afford the synergistic fungicidal action of the both chemicals. Further, there is a possibility that 8-quinolinol makes it possible for copper ion to pass through mitochondrial inner membrane of *A. kikuchiana* and inhibit not only 6-phosphogluconate dehydrogenase in extramitochondrial cytoplasm, but also many dehydrogenases in mitochondria.

INTRODUCTION

Black spot is one of the most important diseases of pears in Japan and is caused by a pathogenic fungus, *Alternaria kikuchiana* Tanaka.¹⁾ Copper 8-quinolinolate is widely used for the prevention of black spot. Copper 8-quinolinolate was first discovered as a fungicide by Meyer in 1932²⁾ and found to be a most promising fungicide not only in laboratory but also under various field conditions.³⁾ Copper 8-quinolinolate and 8-quinolinol stimulated the research on the chelation

theory of fungicidal action.³⁾

Concerning the action mechanism of copper ion, many studies have been conducted. Sherman *et al.*⁴⁾ reported that copper ion inhibited amylases of *Aspergillus oryzae* or others. Hopkins *et al.*⁵⁾ revealed that succinate dehydrogenase and lactate dehydrogenase of rabbit muscle was inhibited by copper ion. Scott *et al.*⁶⁾ revealed that Hg, Zn or Cu ion inhibited 6-phosphogluconate dehydrogenase (Cu, Zn > Hg) and glucose 6-phosphate dehydrogenase (Zn > Hg > Cu) of *Escherichia coli*. Veeger *et al.*^{7,8)} showed that lipoamide de-

hydrogenase of pig heart was inhibited by copper ion and Wren *et al.*^{9,10)} showed that lipoamide dehydrogenase of *Saccharomyces cerevisiae* was also inhibited by copper ion. Most of these studies revealed that copper ion inhibits dehydrogenases, and fungi recover from the inhibition when SH-compound such as glutathione is added.

Toyoda¹¹⁾ studied on action mechanism of copper ion against starved cell of *A. kikuchiana*. The inhibitory effect of copper ion on reduction of triphenyltetrazolium chloride was larger in glucose, glucose 6-phosphate or 6-phosphogluconate solution than in xylose or succinate solution. Therefore, copper ion was considered to act mainly on 6-phosphogluconate dehydrogenase. The inhibitory effect of copper ion was enhanced by the coexistence of 8-quinolinol because permeability of biomembrane to copper ion became higher.

In this report, germination and penetration stages of *A. kikuchiana* were observed on the alcohol-treated epidermal strips of an onion bulb¹²⁾ and the inhibitory effects of copper sulfate, 8-quinolinol and copper 8-quinolinolate on the growth and the respiration of *A. kikuchiana* were studied.

MATERIALS AND METHODS

Sterile distilled water was poured onto an apricot medium on which spores of *A. kikuchiana* were formed. The surface was rubbed with a soft brush and the spore suspension was diluted to give an appropriate density (about 30 spores in a field of 150×microscope magnification). Alcohol-treated onion epidermal strips were inoculated with 20–30 μ l of the suspension of *A. kikuchiana* spores using a microselectapette. They were incubated at 28°C, and spore germination and hyphal penetration were observed at constant intervals with an optical microscope. Effects of copper sulfate, 8-quinolinol and copper 8-quinolinolate were tested with spore suspension adjusted to pH 6 by m/75 phosphate buffer on epidermal strips. After 24 hr incubation, spore germination and hyphal penetration were observed.

Oxygen consumption of mitochondrial fraction of *A. kikuchiana* or rat liver was observed polarographically with a platinum oxygen electrode.

RESULTS

Germination and penetration rates were observed at 2 hr intervals for 24 hr (Fig. 1). A few spores germinated 2 hr after inoculation. Germination rate reached to about 90%, 6 hr, and about 100%, 14 hr after inoculation. A few hyphae penetrated 6 hr after inoculation. Penetration rate reached to about 80%, 16 hr, and about 100%, 24 hr after inoculation. Germination usually occurred from 2 cells of a spore (Plate I–1,2). Hyphae usually penetrated at more than one spot (Plate I–3). Ramifications of penetrated hyphae were often observed (Plate I–3, 4). In these respects, *A. kikuchiana* was different from *Diaporthe citri* which usually germinated and penetrated at one point.¹²⁾ Spore germination, hyphal elongation, hyphal penetration and hyphal growth after the penetration of *A. kikuchiana* were successfully observed on epidermal strips of onion bulb, therefore, the method seemed useful for observation of fungicidal effects on the infection stages of *A. kikuchiana*.

I_{50} of copper sulfate, 8-quinolinol and copper 8-quinolinolate on germination of *A. kikuchiana*

Fig. 1 Time courses of spore germination and hyphal penetration of *Alternaria kikuchiana* on epidermal strips of onion bulb.

○: spore germination, ●: hyphal penetration.

Table 1 I_{50} of copper sulfate, 8-quinolinol and copper 8-quinolinolate against *Alternaria kikuchiana*.

Stage	I_{50} (M)		
	Copper sulfate	8-Quinolinol	Copper 8-quinolinolate
Germination	10^{-3}	10^{-4}	10^{-6}
Penetration	3×10^{-4}	3×10^{-5}	10^{-6}

Table 2 I_{50} of copper sulfate in the presence of 8-quinolinol and that of 8-quinolinol in the presence of copper sulfate against *Alternaria kikuchiana*.

Stage	I_{50} (M)			
	Copper sulfate in the presence of 8-quinolinol at		8-Quinolinol in the presence of copper sulfate at	
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Germination	10^{-3}	2×10^{-6}	7×10^{-6}	2×10^{-5}
Penetration	3×10^{-4}	10^{-6}	—	—

were 10^{-3} M, 10^{-4} M and 10^{-6} M respectively. Those on hyphal penetration were 3×10^{-4} M, 3×10^{-5} M and 10^{-6} M respectively (Table 1). I_{50} of 8-quinolinol and copper sulfate on spore germination were about 10^2 and 10^3 times as high as that of copper 8-quinolinolate respectively, and I_{50} of those on hyphal penetration were 3×10 and 3×10^3 times as high as that of the latter respectively. The result clearly, indicates that copper ion and 8-quinolinol acted synergistically against *A. kikuchiana*. An addition of 8-quinolinol at 10^{-7} M did not influence the inhibitory effect of copper sulfate but greatly enhanced the latter when added at 10^{-6} M, decreasing the I_{50} down to 2×10^{-6} M (Table 2). Copper sulfate inhibited germination, hyphal elongation and penetration at lower concentrations than copper 8-quinolinolate did when 10^{-5} M 8-quinolinol was added

(Table 3). In this case, germination rate decreased as copper concentration changed from 5×10^{-9} M to 2×10^{-8} M, increased from 2×10^{-8} M to 1×10^{-7} M and again decreased from 1×10^{-7} M to 5×10^{-7} M. Germination was completely inhibited at more than 5×10^{-7} M. Hyphal length and penetration rate also changed according to the varying copper concentrations, as germination did (Table 3). On the other hand, I_{50} of 8-quinolinol on germination was 7×10^{-6} M when 10^{-5} M copper sulfate was added, and 2×10^{-5} M when 10^{-4} M copper sulfate was added (Table 2). Therefore, the synergism by copper ion and 8-quinolinol became smaller when copper ion existed more than a half of 8-quinolinol.

We observed the effects of copper sulfate and 8-quinolinol on respiration of the mitochondrial fraction of *A. kikuchiana*. Respiratory control of the mitochondrial fraction was almost lost and R.C. ratio was only 1.2. Copper sulfate inhibited respiration completely at 10^{-3} M. 8-Quinolinol accelerated respiration to 1.4 times at 2×10^{-3} M (Fig. 2).

We then studied the effect of 8-quinolinol on respiration of rat liver mitochondria. R.C. ratio of the mitochondria was 6.4 to 6.7 and ADP/O ratio was 1.7. 8-Quinolinol was a very weak uncoupler and was active in the order of 10^{-3} to 10^{-2} M. It accelerated state 4 respiration to 1.8 times at 5×10^{-3} M and to 2.5 times at 10^{-2} M. ATP production was inhibited at 10^{-2} M as was indicated by H^+ concentration change (Fig. 3). A possibility was suggested that the inhibitory effect of 8-quinolinol on the growth of *A. kikuchiana* was due to the uncoupling effect.

Table 3 Inhibitory effect of copper sulfate in the presence of 10^{-5} M 8-quinolinol against *A. kikuchiana*.

Cu^{2+} (M)	% ^{a)}		
	Germination	Hyphal elongation	Penetration
5×10^{-10}	100	100	74
1×10^{-9}	100	100	65
2×10^{-9}	100	50	29
5×10^{-9}	99	50	29
1×10^{-8}	76	10	5
2×10^{-8}	54	5	34
5×10^{-8}	86	10	50
1×10^{-7}	92	20	60
2×10^{-7}	39	5	0
5×10^{-7}	0	0	0

^{a)} Control=100.

Fig. 2 Influence of 2 mM 8-quinolinol on respiration of mitochondrial fraction of *A. kikuchiana*.

1. Mitochondrial fraction in a suspension medium (0.18 M mannitol, 60 mM sucrose, 10 mM succinate·2Na, 9 mM Tris-buffer, 8 mM phosphate-buffer; pH 7.3) was added.
2. 2 mM 8-Quinolinol was added.
3. 10 mM ADP-Mg was added.

Fig. 3 Influence of 10 mM 8-quinolinol on state 4 respiration of rat liver mitochondria.

1. 0.7 mg/ml mitochondria in a suspension medium (0.2 M sucrose, 2 mM MgCl_2 , 1 mM EDTA·2Na, 10 mM succinate·2Na, 10 mM K-phosphate buffer; pH 7.4) was added with 1 $\mu\text{g}/\text{mg}$ ·protein of rotenone.
2. 200 μM ADP was added.
3. 10 mM 8-Quinolinol was added.
4. 200 μM ADP was added.

DISCUSSION

Copper sulfate inhibited respiration of mitochondrial fraction of *A. kikuchiana*. Restraint on electron transport system by the inhibitory effect of copper ion on dehydrogenases⁵⁻¹⁰⁾ was presumed. 6-Phosphogluconate dehydrogenase exists in a soluble portion of extramitochondrial cytoplasm because it is an enzyme of pentose phosphate pathway and not of TCA cycle.¹⁸⁾ This might be one of the reasons that copper ion strongly inhibits 6-phosphogluconate dehydrogenase of *A. kikuchiana*.

Uncouplers are generally lipid-soluble substances containing an acidic group and can promote a passage of H^+ ions through mitochondrial membrane.¹⁴⁾ Both neutral and anionic forms of an uncoupler must be soluble and stable in mitochondrial membrane to act as protonophores.^{15,16)} Mitochondrial membrane is thus generally permeable to uncouplers and fungal cell membrane which is similar to mitochondrial membrane by nature may also be permeable to 8-quinolinol and permit copper ion to pass the barrier along with 8-quinolinol as a complex to afford the synergistic fungicidal action of the both chemicals.

The permeability of inner membrane of fungal mitochondria may also be very important because copper ion is supposed to be an inhibitor of dehydrogenases and most dehydrogenases exist in inner membrane or in matrix of mitochondria. Mitochondria consists of four parts i.e., matrix, inner membrane, intermembrane space and outer membrane, and peculiar enzymes exist in each part. Enzymes of TCA cycle are mostly in matrix, although succinate dehydrogenase is in inner membrane. To many solutes, outer membrane is permeable but inner membrane is not. In mitochondria, pyruvate is completely oxidized and an electron is transported from succinate or NADH (NADPH) to oxygen ($1/2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}$), producing ATP from ADP and P_i . Liposoluble dehydrogenases like NADH dehydrogenase or succinate dehydrogenase exist inside (matrix side) of the inner membrane^{17,18)} and water soluble dehydrogenase like isocitrate dehydrogenase, glutamate dehydrogenase or malate dehydrogenase exist in

matrix. Copper ion may inhibit not only 6-phosphogluconate dehydrogenase in extra-mitochondrial cytoplasm or some dehydrogenases outside (outer membrane side) of the inner membrane¹⁷⁾ but also some dehydrogenases which exist inside of the inner membrane and in matrix of mitochondria in the presence of 8-quinolinol.¹⁹⁾

When 8-quinolinol existed more than twice of copper ion, the synergism was more remarkable (Table 1, 3). Therefore, not only the translocation of copper ion through cell membrane but also through mitochondrial membrane or the combination of fungicides which has different action points might be concerned with the synergism. A very small

amount of copper ion can inhibit the growth of *A. kikuchiana*, when 8-quinolinol exists in excess and copper ion can reach the action point easily or when some metabolic cycles are inhibited by the effect of 8-quinolinol.

On the contrary, the synergism became less remarkable when copper ion existed more than a half of 8-quinolinol. The reason may be that the amount of 1 : 2 complex of copper and 8-quinolinol decreases and the permeability in biomembrane is in the order of, copper ion < 1 : 1 complex < 1 : 2 complex (copper 8-quinolinolate), or 8-quinolinol can not act well as a protonophore or a chelator with essential metals when copper ion exists in excess.

Plate 1

1. Spore germination from 2 cells, 24 hr after inoculation. Bar: 20 μ m.
 2. Spore germination from 2 cells and hyphal penetration, 24 hr after inoculation. Bar: 40 μ m.
 3. Hyphal penetration from 2 points, 24 hr after inoculation. Bar: 20 μ m.
 4. Ramification of penetrated hypha, 24 hr after inoculation. Bar: 20 μ m.
- hp: hyphal penetration, pm: penetrated hyphae, s: spore.

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要 約

8-ヒドロキシキノリン銅の *Alternaria kikuchiana* に対する作用機構

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A. kikuchiana の孢子発芽, 菌糸伸長, 菌糸侵入の各過程がアルコール処理したタマネギりん片上皮上で観察された。*A. kikuchiana* の発芽に対する硫酸銅, 8-quinolinol および 8-ヒドロキシキノリン銅の 50% 阻害濃度はおのおの 10^{-3} M, 10^{-4} M そして 10^{-6} M であった。侵入に対してはおのおの 3×10^{-4} M, 3×10^{-5} M そして 10^{-6} M であった。したがって, 銅イオンと 8-quinolinol の間に相乗効果が見られた。8-quinolinol が銅イオンの 2 倍以上存在すると, 相乗効果はより大きくなり, 反対に, 銅イオンが 8-quinolinol の 1/2 以上存在すると, 相乗効果は小さくなった。8-quinolinol は, 10^{-2} M でラットのミトコンドリアの state 4 の呼吸を 2.5 倍に促進し, 10^{-3} M 以上で酸化的リン酸化に対して脱共役作用を示した。8-quinolinol は, さらに, *A. kikuchiana* のミトコンドリア分画の呼吸を, 2×10^{-3} M で 1.4 倍に促進することから, 8-quinolinol の *A. kikuchiana* の生育に及ぼす阻害作用に脱共役作用が関与している可能性が示された。ミトコンドリア膜は脱共役剤を透過するので, 性格が類似した真菌類の細胞膜は, 8-quinolinol を透過し, 8-quinolinol は銅イオンとキレート化合物を作ることによって, 銅イオンが細胞膜を通して細胞内へ侵入するのを容易にし, 相乗効果を発揮するものと考えられる。さらに, 8-quinolinol は, 銅イオンが *A. kikuchiana* のミトコンドリア内膜を通してその内側へ到達することを可能にし, 銅イオンは, ミトコンドリア外の細胞質中に存在する 6-ホスホグルコネート脱水素酵素のみでなく, ミトコンドリア内の多くの脱水素酵素をも阻害する可能性がある。