Effect of Polyoxin on Fungi III Electron Microscopic Observation of Mycelia of *Alternaria kikuchiana* Tanaka

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石崎 寛・光岡菊郎・河野 満・久能 均：糸状菌に対するポリオキシンの
影響 III ナシ黒斑病菌菌糸の電子顕微鏡的観察

Abstract

The effects of polyoxin on sensitive and resistant isolates of *Alternaria kikuchiana* Tanaka were examined at the ultrastructural level. The most remarkable change in polyoxin-treated mycelia of the sensitive isolate was the formation of intrahyphae. The inner region of the cell wall of the original hypha expanded inwards and divided the cytoplasm of the parent hypha into several parts, resulting in the formation of intrahyphae. The cytoplasm of the parent hypha degenerated, whereas that of the intrahypha looked normal. The intrahypha often split the cell wall of the parent hypha and expanded outwards through this split, forming a bulbous structure outside the parent hypha. The content of the bulbous structure appeared normal. The hypha of normal appearance further elongated from the bulbous structure or the intrahypha quite often. The elongation of hyphae from these structures is considered to explain one of the actions that polyoxin acts on this fungus as a fungistat rather than a fungicide. In the resistant isolate, similar mycelial changes were observed but frequency of these changes was much less than in the sensitive one.

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Introduction

Polyoxins have unique effects on fungi. Eguchi *et al.*²⁾ reported that all components of the polyoxin complex caused abnormal bulbous structures on germ tube of spore and mycelium of *Alternaria kikuchiana*, causal fungus of the black spot disease of Japanese pear. It has been generally accepted that polyoxin inhibits chitin synthesis in the fungal cell wall and therefore is effective on fungi which have chitin as a cell wall component^{1,3,7)}.

Ishizaki *et al.*⁶⁾ observed that polyoxin treatment caused a bulbous structure to form the germ tubes of *A. kikuchiana* which were surrounded by a two-layered cell wall. If polyoxin was absent, only one layer was observed around the germ tube. In the previous paper describing microscopic observations⁵⁾, we reported that the effects of polyoxin on mycelia of *A. kikuchiana* could be classified into four types, i) induction of a characteristic bulbous structure, ii) induction of intrahyphae, iii) acceleration of septation, and iv) acceleration of pigmentation. On the basis of the above observations, we suggested that polyoxin exerted several effects on this fungus in addition to the inhibition of synthesis of cell wall components. In the present paper we

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studied morphological alterations in polyoxin-treated mycelia of *A. kikuchiana* in more detail at the ultrastructural level.

Materials and methods

A polyoxin-sensitive isolate of *A. kikuchiana* Tanaka (A1-4, the stock number of Mie Univ.) and a polyoxin-resistant isolate (A1-6) were cultured on potato-glucose agar at 28 C for two weeks. A flask with 100ml of liquid potato-glucose medium was inoculated with a fungal disc which was obtained from the above culture medium with a cork borer and incubated at 28 C on a rotary shaker. After three days, 10 mg of Polyoxin-A1 Wettable Powder (Nihon Nohyaku Co. Ltd.) was added into the flask and the flask was further incubated on the shaker. The culture medium contained 10 ppm of polyoxin B equivalents. The flask into which no polyoxin was added served as a control. Two days after adding polyoxin, mycelia of both the isolates were taken out from the flask and fixed with para-formaldehyde plus glutaraldehyde, followed by 1% osmic acid. Control mycelia were fixed at the fifth and ninth days after incubation. They were dehydrated through an ethanol series and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead nitrate and observed with a JEOL-7A electron microscope.

Results

1. Untreated mycelia: The appearance of mycelial cells was very similar in both the isolates. The cell wall surrounded a cytoplasm. The plasma membrane was closely adpressed to the inner cell wall and appeared wavy (Figs. 1, 2). Many mitochondria, abundant ribosomes, endoplasmic reticula, vacuoles and lipid bodies were observed in the cytoplasm (Figs. 1-3). One to three nuclei were present in each cell of the hypha (Figs. 2, 3). Old mycelia which were cultured for nine days were divided into a number of cells by many septae (Fig. 3). The cytoplasm of such old hyphae were very often vacuolized (Fig. 4).

2. Polyoxin-treated mycelia: The polyoxin treatment resulted in conspicuous abnormal growth of the mycelia. Some of the mycelia contained large vacuoles in the cytoplasm. However, in such mycelia the other organelles looked normal (Fig. 5). The most prominent change of the mycelia was the formation of intrahyphae. The content of most hyphae was separated by newly-formed inner cell walls. Accordingly, many independent cells were formed in the original hypha. The cells appeared to be a hypha which was formed inside the original hypha. We propose to call them intrahyphae (Figs. 6-8). The cytoplasm of the intrahypha was surrounded by a continuous cell wall which obviously expanded from the cell wall of the parent hypha (Figs. 6, 7). The cytoplasm of the parent hypha degenerated but that of the intrahypha remained normal (Figs. 6, 7). The intrahypha sometimes split the cell wall of the parent hypha and expanded outwards through this split, forming a bulbous structure outside the parent hypha (Fig. 8). The content of the cytoplasm in the bulbous structure appeared normal, although a large vacuole sometimes occupied the cytoplasm.

Patterns of the abnormal growth of mycelia were very similar in both the isolates. Abnormal growth such as intrahypha and bulbous structures, appeared more

frequently in the polyoxin-sensitive isolate than in the resistant one.

Discussion

In the previous paper⁵⁾ we pointed out that polyoxin induced intrahyphae and bulbous structures in mycelia of *A. kikuchiana*. The present ultrastructural observations supported these conclusions. The inner region of the hyphal cell wall expanded inwards and divided the cytoplasm into several parts, resulting in intrahyphae in the parent hypha. The cytoplasm of the parent hypha appeared degenerate, while that of the intrahyphae was observed to be normal. The content of bulbous structures was also normal. As revealed by light microscopic observations⁵⁾, there were many cases that intrahyphae split the cell wall of the parent hypha and expanded further through this split and that new hyphae originated from bulbous structures. Ishizaki *et al.*⁴⁾ reported that polyoxin, a kind of fungistatic antibiotics, did not completely inhibit mycelial growth of isolates A1-4 and A1-6. Further elongation of intrahypha and newly-born hypha of the compact cytoplasm from bulbous structures are considered to explain such an action of polyoxin.

One of the purposes of the present study was to find differences in polyoxin-sensitive and resistant isolates at the ultrastructural level. However, no significant difference in this regard could be found.

Ishizaki *et al.*⁴⁾ reported that mycelial growth of polyoxin-sensitive isolates was repressed by the polyoxin treatment, whereas polyoxin accelerated mycelial growth of resistant isolate at some growth stage. At the present time we can not explain such a difference between the two isolates in cytological terms. Further physiological and genetic studies should be done to resolve this problem.

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和文摘要

糸状菌に対するポリオキシンの影響 III

ナシ黒斑病菌菌糸の電子顕微鏡的観察

石崎 寛・光岡菊郎・河野 満・久能 均

ナシ黒斑病菌 *Alternaria kikuchiana* Tanaka のポリオキシン感受性株と耐性株に対するポリオキシン AL 水和剤の影響を電子顕微鏡を用いて検討した。ポリオキシン処理菌糸における最も顕著な変化は、内生菌糸の形成であった。菌糸細胞壁の内層が細胞質内に突出し、親の菌糸の細胞質をいくつかの細胞に分割し、いわゆる内生菌糸を形成した。親の菌糸の細胞質は崩壊したが、内生菌糸の細胞質は正常にみえた。内生菌糸はしばしば親の菌糸細胞壁を突き破り、親の菌糸の外側に膨潤型奇形を形成した。膨潤型奇形の内部は、しばしば大きな液胞によって占められていたが、細胞質そのものは正常に見えた。内生菌糸あるいは膨潤型奇形からさらに正常な内容物をもった菌糸が伸長するのが、多くの場合に観察された。このような菌糸の2次的伸長は、ポリオキシンの本菌に対して、殺菌的よりもむしろ静菌的に作用するということを説明しているように思われる。感受性株と耐性株に対するポリオキシンの影響は、非常に似通っており、細胞学的な差異を認めるに至らなかったが、内生菌糸、膨潤型奇形とも、感受性株により頻度高く現れた。

Explanation of Plate

Abbreviations

cw: cell wall cw₁: cell wall of parent hypha cw₂: cell wall of intrahypha cy₁: cytoplasm of parent hypha cy₂: cytoplasm of intrahypha er: endoplasmic reticulum l: lipid body mt: mitochondrion n: nucleus nl: nucleolus nm: nuclear membrane pm: plasma membrane r: ribosome s: septum v: vacuole

- Fig. 1.** A transverse section of untreated hypha of *A. kikuchiana* (Al-4) cultured for 5 days.
Fig. 2. A longitudinal section of untreated hypha of Al-6 isolate cultured for 5 days.
Fig. 3. A longitudinal section of untreated hypha of Al-6 isolate cultured for 9 days.
Fig. 4. A longitudinal section of untreated hypha of Al-6 isolate cultured for 9 days. The cytoplasm is occupied by many vacuoles of various sizes.
Fig. 5. A longitudinal section of polyoxin-treated hypha of Al-6 isolate. The cytoplasm appears unaltered but a large vacuole occupies the cytoplasm.
Fig. 6. The content of a hypha is divided by transverse and longitudinal septae, resulting in intrahyphae in a parent hypha (polyoxin-treated Al-6). The cytoplasm of the parent hypha (cy₁) degenerates, while that of the intrahypha (cy₂) remains normal.
Fig. 7. Intrahyphae in a parent hypha (polyoxin-treated Al-4). The cytoplasm of the parent hypha (cy₁) degenerates. The cell wall of the intrahypha (cw₂) is obviously continuous with that of the parent hypha (cw₁).
Fig. 8. An intrahypha (right upper) connecting with a bulbous structure (left lower) (polyoxin-treated Al-4). The bulbous structure is occupied by a large vacuole but the other portion of the cytoplasm appears normal.