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Ultrastructural Investigation of Loquat Stems Inoculated with a Strain Harbouring an 85-Megadalton Plasmid and a Cured Strain of *Pseudomonas syringae* pv. *eriobotryae**

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Key words: *Pseudomonas syringae* pv. *eriobotryae*, plasmid, cured and uncured strains, ultrastructure, loquat.

In the previous paper[1], it was reported that the loss of an 85-Md plasmid in *Pseudomonas syringae* pv. *eriobotryae* was always accompanied by some changes in symptoms. The strain with 85-Md plasmid developed symptoms by inducing callus-like tissue within 2 or 3 weeks after inoculation. On the other hand, the cured strains without 85-Md plasmid remained light brown and slightly swollen appearance at the inoculated parts even 50 days after inoculation. Although the margin of the lesion became definite after that time, the typical cankers with callus-like tissue were not induced.

In the present study, the multiplication and structural changes were compared in the loquat stems inoculated with a strain that harboured an 85-Md plasmid or a cured strain for 40 days after inoculation, in order to gain information concerning the role of the 85-Md plasmid.

A derivative strain NAE34P$^+$ that still harboured an 85-Md plasmid and a derivative strain NAE34P$^-$ cured of 85-Md plasmid obtained from *P. syringae* pv. *eriobotryae* NAE34 by subculturing five times at 32°C were used in this experiment. No difference between parental strain NAE34 and the derivative strain NAE34P$^+$ was detected in pathogenicity and other physiological characteristics. Two derivative strains were prototrophs.

To investigate the multiplication of the bacteria in YP medium[1], the fresh suspensions (5 ml) of the derivative strain NAE34P$^+$ and NAE34P$^-$ were cultured at 25°C by shaking. Each 0.1 ml of culture suspension was taken out at 12 hr intervals and serially diluted with sterile distilled water. One-tenth milliliter of each dilution was spread on YP agar medium[1] and incubated at 25°C. Colonies were counted after 2 days of incubation.

Inocula were prepared by suspending bacteria grown on YP agar medium in distilled water to give a concentration of about 10^9 cells/ml. A drop of the bacterial suspension was placed on the growing young stems of loquat and pricked into the stem with a needle. The inoculated plants were placed in a growth chamber maintained at 25°C with a 12 hr photoperiod at 30,000 lux. At intervals of 1, 10, 20, 30 and 40 days after inoculation, the inoculated parts of the stems were cut. The surface of cut stems were sterilized with a 70% ethanol and 3% sodium hypochlorite solution. After rinsing in sterile distilled water, the cut stems were ground in 5 ml sterile distilled water. The suspensions thus prepared were serially diluted and plated on YP agar medium. The plates were incubated at 25°C, and the colonies were counted after 3 days.

For electron microscopy, samples were taken from the surrounding tissue of the inoculated sites at 10, 20, 30 and 40 days after inoculation. These samples were fixed in 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) overnight at 4°C. After fixation, the samples were washed in a phosphate buffer and then post-fixed for 2 hr in 1% osmium tetroxide in a phosphate buffer. The samples were dehydrated in an ethanol series and embedded in Luveak-812. The thin sections were cut with a

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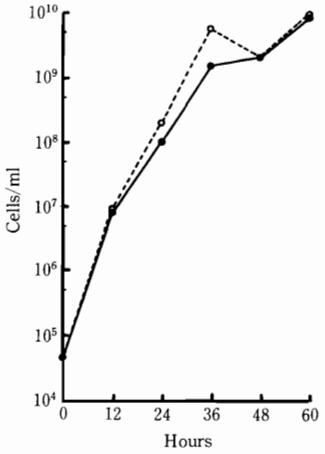

Fig. 1. Growth of the derived strains of *P. syringae* pv. *eriobotryae* NAE34 in the medium. ●: derivative strain NAE34$^+$ that harboured the 85-Md plasmid. ○: derivative strain NAE34$^-$ cured of the 85-Md plasmid.

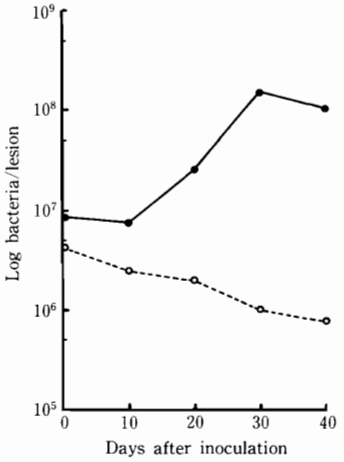

Fig. 2. Growth of the derived strains of *P. syringae* pv. *eriobotryae* NAE34 in the stems of loquat. ●: derivative strain NAE34$^+$ that harboured the 85-Md plasmid. ○: derivative strain NAE34$^-$ cured of the 85-Md plasmid.

ultramicrotome and mounted on collodion-covered grids. The sections were stained with uranyl acetate, followed by lead citrate, and observed in a transmission electron microscope. The ultrastructures of the derivative strains NAE34P$^+$ and NAE34P$^-$ cultured on YP agar medium for 3 days were also compared. The populations of the derivative strains NAE34P$^+$ and NAE34P$^-$ increased rapidly in YP medium. No differences were observed in the growth curve between two derivative strains (Fig. 1). Furthermore, bacterial cells of the two derivative strains were normal in shape (Plate I-1,2).

In the stem tissue of the loquat, the population of the derivative strain NAE34P$^+$ increased from 10 days after inoculation, and reached a maximum of approximately 1.5×10^8 c.f.u. per lesion at 30 days after inoculation (Fig. 2). Symptoms became visible by 20 days after inoculation. The population of the derivative strain NAE 34P$^-$ decreased with time after inoculation, and typical canker symptoms were not produced over the experimental period. The 85-Md plasmid was maintained in all of the eighty colonies reisolated from the lesions induced by the derivative strain NAE34P$^+$. However, the plasmid was not detected in any of the eighty colonies reisolated from the lesions induced by the

derivative strain NAE34P$^-$.

At 10 days after inoculation, the populations of the derivative strains NAE34P$^+$ and NAE34P$^-$ were lower than their initial populations (Fig. 2). In the stem tissue at this stage, similar distorted cells of the derivative strains NAE34P$^+$ and NAE34P$^-$ with dense ribosomes were observed in the intercellular spaces.

At 20 and 30 days after inoculation, the cells of the derivative strain NAE34P$^+$ observed in the intercellular spaces presented normal structural features (Plate I-3). Collapsed and plasmolyzed host cells were frequently observed at 30 days after inoculation (Plate I-3). In the stem of the loquat at 20 and 30 days after inoculation with the derivative strain NAE34P$^-$, two kinds of bacterial cells were observed in the intercellular spaces. One had the normal shape of bacterial cells, but the other had electron-dense and distorted bacterial cells (Plate I-4). The host cells adjacent to the bacterial cells were more or less denatured at this stage, but not collapsed (Plate I-4). The cells of NAE34P$^+$ and NAE34P$^-$ in the intercellular spaces were surrounded by a small or a large amount of fibrillar material which seemed to be extracellular polysaccharide[2,3]. There was no remarkable difference in quantity and structure of fibrillar material between NAE34P$^+$ and NAE34P$^-$. At 40 days after inoculation with the derivative strain NAE34P$^+$, the collapsed host cells were observed more frequently. Some groups of the derivative strain NAE34P$^+$ adjacent to the collapsed host cells were surrounded by electron-dense granular material, but bacterial cells preserved their relatively normal shape (Plate I-5). Although a large number of bacterial cells of the derivative strain NAE34P$^-$ were observed in the intercellular spaces at 40 days after inoculation, collapsed host cells adjacent to these bacteria were rarely observed (Plate I-6). A large proportion of the cells of the derivative strain NAE34P$^-$ were morphologically irregular (Plate I-7).

Differences were discovered in the development of symptoms between the derivative strain that harboured an 85-Md plasmid and the cured strain. The productivity of plant hormones such as indoleacetic acid (IAA) and cytokinin, which seemed to be important from the point of view of developing symptoms, were investigated. However, there were no significant differences in the ability to produce IAA[1] and cytokinin (unpublished data) between the derivative strains NAE34P$^+$ and NAE34P$^-$. When the derivative strains NAE34P$^+$ and NAE34P$^-$ were inoculated into the stem tissue of the loquat, there was a large difference in the bacterial population between the two derivative strains. In addition, a striking dissimilarity was found in the shape of the cells between the two derivative strains. The observations presented here show that the population of the derivative strain NAE34P$^-$ in the stem tissue decreased and a large number of their cells was distorted, whereas the population of the derivative strain NAE34P$^+$ increased in the stem tissue and the shapes of their cells were almost normal. The failure of multiplication in the stem tissue of the derivative strain which had been cured of the 85-Md plasmid seems to be associated with no canker development. To find the role of the 85-Md plasmid, further studies are needed to determine the reason why the cells fail to multiply in the stem tissue of the loquat.

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和 文 摘 要

上運天 博：ビワがんしゅ病菌の85-Mdプラスミド保有株および欠落株を接種したビワ茎の電顕観察

ビワがんしゅ病菌の85-Mdプラスミド保有株と欠落株のビワ茎組織における増殖および形態的変化を接種40日後まで比較検討した。その結果，保有株は接種10日後から増殖を続け，明らかな病徴を発現した。それに反し，欠落株

の菌数は接種後から減少し，その間明瞭な病徴は認められなかった。なお，両菌株の培地中における増殖能には差は認められなかった。接種10日後の細胞間隙に観察された両菌株間に形態的差異は認められなかった。接種20日および30日後の保有株は正常な形態を有し，隣接宿主細胞は変性あるいは壊死収縮していた。それに対し，欠落株は電子密度の高くなった不整形の菌体が多く観察されるようになり，隣接宿主細胞の壊死は認められなかった。接種40日後，保有株は比較的正常な形態を有していたが，欠落株の多くの菌体は異常な形態を呈し，その形態も多岐にわたっていた。

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Explanation of plate

Plate I

1. Cells of *P. syringae* pv. *eriobotryae* NAE34P$^+$ cultured on the medium. (×12,200)
2. Cells of *P. syringae* pv. *eriobotryae* NAE34P$^-$ cultured on the medium. (×10,800)
3. Normal cells of NAE34P$^+$ and collapsed host cell adjacent to the bacteria. (30 days after inoculation, ×13,700)
4. Normal and distorted electron-dense cells of NAE34P$^-$. Host cells adjacent to these bacteria were hardly affected. (30 days after inoculation, ×10,500)
5. Cells of NAE34P$^+$ surrounded by electron-dense granular material. (40 days after inoculation, ×22,500)
6. Intercellular space occupied by a large number of NAE34P$^-$. (40 days after inoculation, ×4,700)
7. Irregular shaped cells with dense ribosomes of NAE34P$^-$. (40 days after inoculation, ×21,600)

Abbreviations: b, bacterial cell; chc, collapsed host cell; fm, fibrillar material; gm, electron-dense granular material.

Plate I