Isolation of *Rosellinia necatrix* Mutants with Impaired Cytochalasin E Production and Its Pathogenicity

Satoko KANEMATSU*, Tateki HAYASHI* and Akira KUDO**

Abstract

Cytochalasin E (CE) is known to be a secondary metabolite secreted *in vitro* by *Rosellinia necatrix*, the cause of white root rot, and is toxic to plants. In this study, CE was detected in Japanese pear and apple roots infected with *R. necatrix* using a new HPLC method. In order to elucidate the relationship between the productivity of CE and disease progress, CE deficient mutants of *R. necatrix* were obtained by UV irradiation. Two CE-less producing mutants were isolated by a simple TLC detection method. The pathogenicity of these mutant strains was the same as the parent (wild-type) strain. Although, these mutant strains produced CE only about 4-7% level of the parent strain *in vitro*, and less than 5% level *in planta*. The meaning of CE for disease progress of white root rot was discussed.

(Received March 24, 1997 ; Accepted August 7, 1997)

Key words : cytochalasin E, *Rosellinia necatrix*.

INTRODUCTION

Rosellinia necatrix Prillieux is the causal agent of white root rot, and a wide range of fruit trees are killed by this disease^{17,18)}. This fungus infects about 170 species of plants in 63 genera and 30 families^{9,18)} and cross inoculation tests revealed no differences in the virulence of three *R. necatrix* isolates obtained from different host species¹⁹⁾. In spite of its large economic damage to fruit production, there is little effective method of protection against this disease. Pathogenesis factors of *R. necatrix* are complex and not well understood, although various secondary metabolites are toxic to plants^{2-4,10,11)}. Many workers identified cytochalasin E (CE, Fig. 1) amongst metabolites extracted from the culture filtrate of *R. necatrix*^{2,10,16)}, and it is thought that CE acts as a toxin during disease progress. But it has not yet been shown that CE is produced also *in vivo*, and the relationship if any, between pathogenicity and productivity of CE in this fungus is still unclear.

In this paper, we report that CE was secreted onto roots infected with *R. necatrix*. With the aim of understanding better the role of CE in the pathogenicity of the fungus, we have followed the classical strategy of producing CE deficient mutants of *R. necatrix*, and determined the pathogenicities of these mutants.

MATERIALS AND METHODS

HPLC of CE extracted from infected roots

CE was isolated by the method of Kimura *et al.*¹¹⁾ with some modifications. An acetone extract of infected Japanese pear or apple roots was chromatographed twice on silica gel (silica gel 60 ; Merck) column (10 × ϕ 1.6 cm) with benzene-acetone (4 : 1, v/v). The fraction corresponding to CE was concentrated *in vacuo*. A new HPLC method was used for identification of CE. The residue was injected into a ODS column and separated using a solvent system consisting of 65%-90% methanol gradient containing 1% acetic acid. The peak of CE was detected by UV (A_{280}) and its identity confirmed by comparing with the retention time of an isolated sample with one mixed with authentic material (Sigma, Cat No. C2149).

NMR analysis of CE from mycelial mats

The NMR spectrum of the CE crystal derived from cultured mycelial mat of *R. necatrix* was measured. In order to obtain CE crystals, *R. necatrix* R-24 (wild-type strain) was cultured on MPD broth (see later) at 25°C for 3 weeks, and then the mycelial mat dried and extracted with acetone. From this extract, CE was purified using column chromatography described above, only the scale (column size ; 20.5 × ϕ 5 cm) was different. The dried residue was dissolved to acetone, and mixed with hexane until the crystal began to develop. ¹H-NMR spectrum was measured by dissolving the crystal in acetone-

* National Institute of Fruit Tree Science, MAFF, Tsukuba 305, Japan 農林水産省果樹試験場

** Present address : National Institute of Fruit Tree Science, MAFF, Citrus Division, Kuchinotsu, Nagasaki 859-25, Japan
現在 : 農林水産省果樹試験場カンキツ部

Fig. 1. Chemical structure of cytochalasin E.

d_6 containing 1% TMS with a JEOL JNM-PMX60SI.

Microorganisms The wild-type *R. necatrix* strain R-24 was used for inducing mutants. R-24, isolated from a diseased mulberry root, was kindly supplied by Dr. H. Ieki (National Institute of Fruit Tree Science).

Media Potato dextrose broth (PDB) and potato dextrose agar (PDA) medium (Difco) were used for the preparing mycelial fragments and regeneration colonies, respectively. For screening of CE production on micro tube culture, MPD medium containing 2% malt extract (oxid), 0.1% peptone (Difco), 2% dextrose was used.

Mutagenesis procedure Mycelial mats were obtained from a shaking culture of the wild-type strain grown in 50 ml PDB at 25°C for 4 days. The mycelia were aseptically homogenized at 13,000 rpm for 2 min and fragments filtered through three layers of gauze, and diluted 10 times with distilled water. For inducing mutation, these mycelial fragments (less than 100 μ m long) were exposed with stirring in a petri dish to short wave-length (254 nm) UV light at a distance of 30 cm from the lamp (15 W) for 60 sec (less than 1% viability). Mycelial suspensions (0.3 ml) were spread on PDA medium for colony regeneration. These plates were incubated at 25°C for 8 to 10 days, and each colony (approximately 2 mm $\times$ 2 mm in size) was then transferred to a micro-tube containing 0.8 ml of MPD medium, and grown at 25°C for 9 days. After cultivation, all of the contents (mycelia and media) in each micro-tube were dried *in vacuo*, and extracted with 0.7 ml of acetone. This acetone extract (160 μ l) was used for CE detection after separation by TLC on silica gel.

Detection method for CE using TLC Epoxide detection of oxidized oil on TLC⁷⁾ was used with some modifications. An acetone extract of the micro tube culture was spotted directly on a silica gel plate (silica gel 60; Merck), dried and developed with acetone-hexane (2:3, v/v). The plate was sprayed thoroughly with 0.05 M picric acid in 95% ethanol and immediately placed in a tank saturated with the vapor of diethyl

Table 1. Amount of cytochalasin E contained in culture filtrate of mutant and wild-type strains

Strain	Amount of CE ^{a)} (μ g of CE/ml of culture filtrate)	Dry weight of mycelia ^{a)} (mg)
R-24 ^{b)}	18.1 $\pm$ 2.1	193 $\pm$ 18.6
M1 ^{c)}	0.7 $\pm$ 0.5	157 $\pm$ 14.3
M2 ^{c)}	1.2 $\pm$ 1.1	224 $\pm$ 20.9

a) Results are represented as the mean of three independent assays followed by the standard deviation.

b) Wild-type strain.

c) Mutant strain.

ether-95% ethanol-acetic acid (80:20:1, v/v/v). More than 30 min later, the plate was taken out and exposed to ammonia fumes for 3 min.

To confirm the ability of this procedure to detect CE, materials which have similar structure to CE were tested as references: cytochalasin B (Sigma, Cat No. C6762), no epoxide position on its structure; chaetoglobosin C (Sigma, Cat No. C1282) and rosellichalasin (kindly given by Dr. Y. Kimura, Tottori University)¹¹⁾ which have an epoxide group.

Quantification of CE in the extracts of roots infected with mutant and parent strains In order to determine the production of CE *in planta*, fresh root (0.1 g) of wild pear strain Mamenashi No. 6 was placed on a PDA culture plate of either mutant or parent strains, incubated at 25°C in the dark. Roots were sampled at regular intervals and extracted with acetone after drying *in vacuo*. All the acetone extract was spotted on a Silica gel plate, and CE detected by the method described above. The amount of CE detected by TLC was measured with a densitometer (Shimazu, CS-9300PC).

Quantification method for CE by HPLC CE was also quantified by HPLC using almost same method utilized for detecting CE from infected roots. A mycelial plug, 4 mm in diameter, and grown on PDA was statically cultured in 25 ml MPD medium in 100 ml flasks at 25°C for 13 days. After cultivation, both culture filtrate (10 ml) and mycelia were collected separately on a glass fiber filter (ADVANTEC, GA100). Each of them was lyophilized, and extracted with acetone. This extract was chromatographed once on a silica gel column with benzene-acetone (4:1, v/v). The eluate was concentrated *in vacuo*, and fractionated by HPLC using a solvent system 65% methanol containing 1% acetic acid. Other HPLC conditions were same as these used to extract CE from infected roots.

Evaluation of *in vitro* growth rate and colony characteristics The culture method described in "Quantification method for CE by HPLC" was used for the growth-rate assay. A mycelial mat was washed with distilled water, and dried at room temperature, and the dry weight of mycelia measured. For the characterization of colony morphology, cultures were examined after incubation at 25°C for 2 weeks in the dark.

Pathogenicity test Two types of inoculation test (A and B) were carried out at 25°C in a greenhouse. In both tests, seeds or seedlings of the wild pear strain Mamenashi No. 6 were used as the assay plant. Test A was "soil-over-agar-culture-inoculation-method"^{20,21)} with the following minor modifications. Mutant or wild-type strains were cultured on PDA in a petri dish (3 cm depth, 9 cm in diameter) at 25°C for 10 days. Then, the colony was covered with about 50 ml of autoclaved soil (soil : sand, 2 : 1, v/v) and 10 seeds sterilized with antiformin were sown on the soil, and covered with additional 50 ml of autoclaved soil. Five replicated dishes were used for each strain.

In test B, 6-month-old seedlings were used for inoculation. Each strain was grown on autoclaved Japanese pear twig cuttings (30 mm length, 10 mm in diameter) at 25°C for 13 days. One seedling was planted into autoclaved soil containing 10 g of inoculated twigs in 11 cm diameter pot. Five replicated pots were used for each strain.

RESULTS

Identification of CE in infected roots and mycelial mats

The chromatographed CE fraction from the extracted mycelial mats showed one high peak on HPLC (Fig. 2), and this peak had the same retention time (22 min) as authentic CE. From 3 g of dried mycelial mats, 14.9 mg of CE crystal was obtained. Because the NMR spectrum of this crystal was identical to the authentic CE (Fig. 3), this peak (retention time: 22 min) on HPLC was

Fig. 2. HPLC profiles (A_{280}) of acetone extract from mycelial mats of *R. necatrix* (A) and apple roots infected with *R. necatrix* (B).

identified as CE. The same peak from the extract of infected Japanese pear and apple roots on HPLC was also identical to authentic CE (Fig. 2). This same peak could not be detected in extracts of healthy roots. The amounts of CE detected in this experiment were 35 μ g and 17 μ g per 1 g of dried roots of Japanese pear and apple, respectively.

Development of Detection method for CE on TLC

Acetone extract from mycelial mats of *R. necatrix* resulted in the appearance of too many spots to identify CE on a TLC plate when the non-selective standard method was used⁵⁾, in which the plate was sprayed with sulfuric acid and heated. So it was very difficult to judge which spot was derived from CE. In contrast, only one

Fig. 3. ¹H-NMR spectra of authentic CE (A), and CE crystal derived from mycelial mats of *R. necatrix* (B). This crystal was dissolved in acetone d_6 containing 1% TMS.

Fig. 4. TLC of cytochalasin B (CB, 60 μ g), chaetoglobosin C (ChC, 60 μ g), cytochalasin E (CE, 5 μ g) and rosellichalasin (RC, 50 μ g). This plate was developed with acetone : hexane (2 : 3), and treated with picric acid.

spot of CE appeared on a TLC plate by using newly developed epoxide method. In this procedure, CE (R_f : 0.36), rosellichalasin¹¹⁾ (R_f : 0.47), and chaetoglobosin C⁵⁾ (R_f : 0.39), all of them have an epoxide, could be detected. The orange spot of chaetoglobosin C was so unstable as to disappear immediately when the plate was taken from the tank saturated with ammonia vapor. On the other hand, cytochalasin B⁵⁾, which has no epoxide position in its structure, showed no spots on a TLC plate (Fig. 4). CE appeared as a blue-purple spot in about 30 min after treatment of the TLC plate with ammonia fumes and remained for more than 2 days.

Selection of mutants

Two CE deficient mutants, M1 and M2 were obtained from 400 surviving mycelial fragments of UV irradiated *R. necatrix*. They were identified from the point of view of CE production by the TLC method (Fig. 5). M1 produced a blue-purple spot different from CE at R_f 0.43. From these mutants, single hypha isolation was

repeated three times and strains thus obtained were used for further experiments.

Characterization of the mutants

CE deficient mutants selected by the TLC method were further evaluated for CE productivity *in vitro* by HPLC. In order to speculate the CE amount secreted by the fungus on the roots, acetone extracts of lyophilized liquid culture after growth on MPD were used. The growth rate in culture of mutants on MPD medium was almost same level as the wild-type strain after incubation for 13 days. Mutants (M1 and M2) produced only 0.7 and 1.2 μg of CE/ml of culture filtrate, respectively, although the wild-type strain (R-24) produced 18.1 $\mu\text{g}/\text{ml}$ in the same condition (Table 1). At the same time, in the acetone extract of mycelia of R-24 and M2, CE was detected 43 and 5 $\mu\text{g}/0.1\text{ g}$ of dried mycelia, respectively. But it was not detected in the extract of M1.

In order to determine the production of CE *in planta*, roots of a wild pear strain Mamenashi No.6 were placed on PDA culture plates of each fungal strain (Fig. 6). One day after inoculation, the root was slightly infected at the bottom surface attached to the culture plate. At this stage CE was detected only in the extract of the root inoculated with R-24. After 3 days, roots were thoroughly infected with mutant or parent strains. CE was not detected in the extract of M1, though M2 produced CE at a level of about 4% of that of R-24. After 5 days incubation, the amounts of CE in the extracts of the roots infected with mutant (M1 and M2) and parent (R-24) strains were 0.1, 0.4 and 14.2 $\mu\text{g}/0.1\text{ g}$ of fresh root, respectively. M1 produced a different spot (R_f : 0.43) from that of CE on TLC, the same spot was also detected in the extract of the M1 culture (Fig. 5, 6).

Colony morphology of M1, M2 and R-24 was typical of *R. necatrix*, hyphae of both mutants showed pear shape swellings at the site of septa.

Pathogenicity test

CE-less producing mutants were pathogenic to roots

Fig. 5. TLC of the acetone extracts of the wild-type strain R-24, and CE deficient mutants M1 and M2 of *R. necatrix*. Each lane was spotted with 160 μl of acetone extracts, and the left, 5 μg of CE was spotted as a marker.

Fig. 6. Roots of wild pear strain Mamenashi No. 6 infected with mutant (M1 and M2) and parent (R-24) strains on PDA culture plates after 1, 3 and 5 days incubation (upper row). The presence of CE in each acetone extract was shown by TLC (lower row). An uninfected root placed on a filter paper moistened with water acted as a control. Authentic CE (0.5 μg) was spotted on the right hand lane as a maker.

Table 2. Pathogenicity of mutant and wild-type strains of *R. necatrix* to the potted seedlings of Mamenashi No. 6 (Test B)

Strain	1 wk			2 wk			3 wk			4 wk		
	H ^{e)}	W ^{f)}	D ^{g)}	H	W	D	H	W	D	H	W	D
R-24 ^{b)}	5	0	0	3	2	0	1	2	2	0	0	5
M1 ^{c)}	5	0	0	3	2	0	0	3	2	0	0	5
M2 ^{c)}	5	0	0	4	1	0	1	4	0	0	0	5
Control ^{d)}	5	0	0	5	0	0	5	0	0	5	0	0

a) Data were recorded every week after inoculation.

b) Wild-type strain.

c) Mutant strain.

d) Not inoculated.

e) Number of healthy seedlings.

f) Number of wilted seedlings.

g) Number of died seedlings.

Fig. 7. Damped off seedlings of wild pear strain "Mamenashi No. 6" inoculated with *R. necatrix* mutant strain M2 (A) and wild-type strain R-24 (B), respectively. The C was no inoculation as a control (in Test A).

Fig. 8. Pathogenicity of wild-type strain R-24, CE less-producing mutants M1 and M2 of *R. necatrix* to the wild pear strain (in test A). Disease incidence indicates the mean percentage {(No. of damped off seedlings/No. of seedlings tested) × 100} in 5 replicates.

of the wild pear strain Mamenashi No. 6 at the similar level as the wild-type strain. In test A, one month after inoculation, the roots were infected throughly with these mutant strains (Fig. 7, 8). In test B, same results were obtained. All mutant strains were pathogenic to the seedlings of Mamenashi No. 6 (Table 2, Fig. 9). There

Fig. 9. Wilted seedling inoculated with M1 (the left) and control (the right) (in Test B).

was no difference in disease incidence and speed of disease development between the parent strain R-24 and both mutant strains, M1 and M2.

DISCUSSION

CE has toxicity to plants¹⁶⁾, and was identified from roots infected with *R. necatrix* (Fig. 2). There is a possibility that CE plays an important role in disease progress of white root rot. In order to elucidate any possible relationship between the productivity of CE and virulence of the fungus, we aimed to prepare CE-less producing mutants. First, we needed to develop a simple CE detection method using silica gel TLC for the selection of CE-deficient mutants. The previously reported method to detect CE on a TLC plate⁵⁾ was not suitable for crude extracts of samples, because there are too many spots to identify CE on the plate. CE has an

epoxide group, and so we applied the detection method for an epoxide of oxidized oil on a TLC plate⁷⁾ to detect CE. By using this method, we removed other components and almost quantified CE easily. This method was quite efficient for selection of CE-less producing mutants.

Of the two mutants, M1 and M2, obtained in this experiment, M1 might be a precursor accumulating mutant because M1 produced a different spot from CE and rosellichalasin on a TLC plate *in vitro* and *in planta* (Fig. 5, 6). But the structure and characteristics, including effect to plants, of this product is unclear as yet.

Mutant strains (M1 and M2) produced CE about 4–7% level of the parent strains *in vitro*, and less than 5% level *in planta*. These mutant strains infected roots of a wild pear strain at the same level and speed as the parent wild-type strain, R-24 (Fig. 7, 8, Table 2). This result indicates that amounts of CE produced by *R. necatrix* did not affect disease progress of white root rot. Therefore, CE may not be the main factor of the pathogenicity of *R. necatrix*.

CE acts as a wilt toxin to apple shoots with 4 leaves at 1 to 10 ppm¹⁶⁾. It is well known that cytochalasins act on some types of contractile microfilament systems^{1,6,8,15)}. Recently, it has been suggested that microfilaments of the plant cytoskeletons facilitate plant defence responses to the region of infection^{13,14)}. Using a fluorescent staining method to detect microfilaments¹²⁾, it should be possible to observe if there are some effects of cytochalasins produced by wild and mutant strains on the target cells. This approach may well clarify if the very small amounts of CE produced *in planta* by CE-less producing mutants would be enough to prevent the functioning of the microfilament system.

We wish to thank Dr. K. Yabe, National Food Research Institute, for helpful suggestion, and Dr. H. Ishii, National Institute of Agro-Environmental Sciences, for critical reading this manuscript. We also wish to thank Dr. D.W. Hollomon, IACR Long Ashton Research Station, U.K., for correcting the English.

Literature cited

1. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1994). *Molecular Biology of the Cell*, 3rd ed., Garland Publishing Inc., New York, pp. 823–826.
2. Aldridge, D.C., Burrows, B.F. and Turner, W.B. (1972). The structure of the fungal metabolites cytochalasin E and F. *J.C.S. Chem. Commun.*: 148–149.
3. Chen, Y.S. (1960). Studies on the metabolic products of *Rosellinia necatrix* Berlese. Part I. Isolation and characterization of several physiologically active neutral substances. *Bull. Agric. Chem. Soc. Jpn.* 24: 372–381.
4. Chen, Y.S. (1964). Studies on the metabolic products of *Rosellinia necatrix* Berlese. Part II. The structure of rosellinic acid. *Agric. Biol. Chem.* 28: 431–435.
5. Cole, R.J. and Cox, R.H. (1981). *Handbook of Toxic Fungal Metabolites*, Academic Press, New York, pp. 264–343.
6. Cooper, J.A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105: 1473–1478.
7. Fioriti, J.A. and Sims, R.J. (1968). A spray reagent for identification of epoxides on thin layer plates. *J. Chromatogr.* 32: 761–763.
8. Flanagan, M.D. and Lin, S. (1980). Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* 255: 835–838.
9. Khan, A.H. (1955). *Dematophora* root rot. *Calif. Dep. Agric. Bull.* 44: 167–170.
10. Kimura, Y., Nakajima, H. and Hamasaki, H. (1987). Isolation and biological activity of the fungal metabolite cytochalasin E. *Proceedings of the Plant Growth Regulator Society of America, 14th Annual Meeting*: 51.
11. Kimura, Y., Nakajima, H. and Hamasaki, H. (1989). Structure of rosellichalasin, a new metabolite produced by *Rosellinia necatrix*. *Agric. Biol. Chem.* 53: 1699–1701.
12. Kobayashi, I., Kobayashi, Y., Yamaoka, N. and Kunoh, H. (1991). Recognition of a pathogen and a nonpathogen by barley coleoptile cells. III. Responses of microtubules and actin filaments in barley coleoptile cells to penetration attempts. *Can. J. Bot.* 70: 1815–1823.
13. Kobayashi, I., Kobayashi, Y., Yamada, M. and Kunoh, H. (1996). The involvement of the cytoskeleton in the expression of nonhost resistance in plants. *In Molecular Aspects of Pathogenicity and Resistance: Requirement for Signal Transduction* (Mills, D. *et al.* eds.), APS Press, St. Paul, pp. 185–195.
14. Kobayashi, I., Murdoch, L.J., Kunoh, H. and Hardham, A.R. (1995). Cell biology of early events in the plant resistance response to infection by pathogenic fungi. *Can. J. Bot.* 73 (Supplement 1): S418–S425.
15. Maclean-Fletcher, S. and Pollard, T.D. (1980). Mechanism of action of cytochalasin B on actin. *Cell* 20: 329–341.
16. Sawai, K., Okuno, T. and Ito, T. (1982). The toxicity of cytochalasin E on plants. *Ann. Phytopathol. Soc. Jpn.* 48: 529–531.
17. Sivanesan, A. and Holliday, P. (1972). *In Descriptions of Pathogenic Fungi and Bacteria*, Commonwealth Mycological Institute, Kew, Surrey, England, No. 352.
18. Szejnberg, A. and Madar, Z. (1980). Host range of *Dematophora necatrix*, the cause of white root rot disease in fruit trees. *Plant Dis.* 64: 662–664.
19. Watanabe, B. (1963). Studies on the ecology and control of white root rot disease caused by *Rosellinia necatrix* (Hart.) Berl. *Appointed Experiment (Plant disease and Insect Pests)* 3: 9–11 (in Japanese).
20. Watanabe, T. (1988). Pathogenic fungi associated with forest seeds including *Pythium* species from cherry seeds. *Trans. Mycol. Soc. Jpn.* 29: 197–203.
21. Watanabe, T. (1992). Sporulation of *Dematophora necatrix* *in vitro* and its pathogenicity. *Ann. Phytopathol. Soc. Jpn.* 58: 65–71.

和 文 摘 要

兼松聡子・林 建樹・工藤 晟：白紋羽病菌のサイトカラシン E 産生能変異株とその病原性

サイトカラシン E (CE) は白紋羽病菌によって産生される植物に毒性をもつ二次代謝産物である。本実験において、白紋羽病

菌に感染したリンゴおよびナシの根から CE が検出された。CE 産生能と病原性との関連を調べるため、薄層クロマトグラフィーを用いた CE の簡便な検出法を開発し、本法を用いて UV 照射によって得られた CE 産生能変異株、2 株を選抜した。これらの変異株は野生型株の約 4~7% の CE 産生能しか有していなかったが、マメナシ実生に対して野生型と同等の病原性を示した。