MTT比色定量法を用いたトマト培養細胞に対するAL毒素作用の検定

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Effect of AL-toxin Produced by *Alternaria alternata* Tomato Pathotype on Viability of Cultured Tomato Cells Determined by MTT-Colorimetric Assay*

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Abstract

Effect of AL-toxin produced by *Alternaria alternata* tomato pathotype on viability of cultured cells derived from susceptible and resistant cultivars of tomato was examined. When calli of each cultivar were incubated on solid medium containing the toxin, cell browning occurred only on calli of susceptible cultivar within 3 days after the treatment. For quantitatively estimating the viability of toxin-treated cells, a colorimetric assay utilizing a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was employed. The number of living cells was proportional to the amount of formazan formed by reduction of MTT in active mitochondria, indicating that MTT-colorimetric assay should be useful for the quantitative determination of cell damage induced by the toxin. Formazan production in suspension-cultured cells of susceptible cultivar was inhibited by the toxin at about the same concentration as the minimum concentration for necrosis induction on leaves. In contrast, resistant cells were not affected significantly by the toxin even at higher concentrations. The MTT assay appeared to have some advantages over conventional techniques for evaluating the effect of toxin on cultured cells. Additionally, these results indicate that the host-selective toxicity of AL-toxin is expressed to the cultured cells as well as the plant tissues.

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Key words: *Alternaria alternata* tomato pathotype, AL-toxin, tomato callus, MTT-colorimetric assay.

INTRODUCTION

A stem canker disease caused by *Alternaria* on certain tomato cultivars such as Earlypak 7 was reported from California in 1975[9]. The causal organism was identified to be *A. alternata* f. sp. *lycopersici*, which produced a host-selective toxin (AAL-toxin)[8,9]. The same disease was reported to occur in Japan in 1977[21]: this pathogen infected the cv. First and its derivatives and was designated as *A. alternata* tomato pathotype. The Japanese isolate also produced a host-selective toxin, designated as AL-toxin, which affects the same tomato cultivars as reported for the American isolates[14]. Kohmoto *et al.* concluded that American and Japanese isolates belong to the same pathotype of *A. alternata*[14].

AAL-toxin was purified and characterized as T_A and T_B by Bottini *et al.*[1], and Bottini and Gilchrist[2]. Each toxin consists of two closely related compounds with the same specific activity[20]. Two host-selective toxins, AL-toxin I and II[14], from the Japanese isolates were pu-

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rified and characterized to have almost the same chromatographic behavior as AAL-toxin. However, whether AL-toxin I and II are identical in chemical structure to T_A and T_B remains to be determined.

AL-toxin did not cause a rapid loss of electrolytes from susceptible leaf tissues until necrosis appeared[14]. The first effect of AL-toxin detected by electron microscopy was on mitochondria and rough endoplasmic reticula 24 hr after toxin treatment[18]. However, AL-toxin did not affect respiration by isolated mitochondria from susceptible tomato (unpublished data). On the other hand, Gilchrist[7] indicated that the toxin inhibited the activity of aspartate carbamoyltransferase (ACTase; EC 2.1.3.2.) *in vitro*, and that necrosis formation by the toxin was reduced by treatment with orotate and L-aspartate. Meanwhile, Fuson and Pratt[4] reported that effects of the toxin on tomato cultured cells were inconsistent with ACTase inhibition. Thus, the primary action site for the toxin in host cells is still an open question.

Cultured cells have some advantages over intact plant tissues for the study of physiological effect of toxins: cultured cells can be easily obtained under controlled growth condition and can be uniformly treated with toxins. In general, survival or proliferation of cells have been assayed by measuring weight or volume of cells, by determining plating efficiency, and/or by counting cells after staining with dyes such as FDA[23] and Evans blue[5]. These assay methods have been used for the study of AL-toxin's effect on cultured cells[4,24]. In this study, we developed a simple and rapid colorimetric assay method for quantitative estimation of tomato cell viability. This method is based on the reduction of tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to dark-blue formazan in active mitochondria as demonstrated in living mammalian cells[16]. Brief reports of this study was published previously[11,12].

MATERIALS AND METHODS

Plants and cell cultures. Tomato (*Lycopersicon esculentum* Mill.) cvs. Mie-First and Earlypak 7, susceptible to the canker, and cv. Ace, resistant one, were used. Callus cultures were initiated from 5- to 7-day-old hypocotyls placed onto Murashige-Skoog (MS) medium[17] and incubated for 20 days. MS medium supplemented with 0.2 mg/l 2,4-D and 0.2 mg/l kinetin was used for cvs. Mie-First and Ace, and 0.5 mg/l 2,4-D and 0.15 mg/l kinetin was used for cv. Earlypak 7. The suspension cultures were started from friable calli and maintained in a 125 ml Erlenmyer flask containing 50 ml of MS liquid medium of the same composition as those used for callus induction. The suspension cultures were incubated at 26°C on a gyratory shaker (130 rpm) and subcultured every 14 days.

Toxin purification and leaf bioassay. AL-toxin I was purified from culture filtrates of *A. alternata* tomato pathotype isolate As-27 according to Kohmoto *et al.*[14]. To determine toxic activity of the toxin preparation, a leaf bioassay was used[14]. This toxin preparation caused necrosis on susceptible tomato leaves at concentrations of more than 0.025 μg/ml.

Callus bioassay. To examine toxicity of AL-toxin I to cultured cells, calli of each cultivar were placed on MS solid medium added with the toxin at concentrations of 0.05, 0.5 and 5 μg/ml. After incubation for 3 and 6 days, change in color of each callus was observed. In the case of suspension cell cultures, 450 μl of cell suspensions (1×10^4 cells) were incubated with different amounts of the toxin in 24-well flat-bottomed trays (Falcon). After incubation for 3 and 6 days, cell viability was determined by the methods described below. In some experiments, FDA[23] and Evans blue[5] staining methods were also used for the determination of cell viability.

MTT-colorimetric assay. MTT-colorimetric assay was modified from the method of Mosmann[16]. MTT (Sigma) was dissolved in 0.05 M sodium phosphate buffer (pH 7.5) to give a concentration of 5 mg/ml. After toxin treatment, 50 μl of MTT solution was added

to each well of a 24-well tray containing 450 μl of cell suspension. The plates were incubated for 1 to 6 hr at various temperatures. After incubation, cell suspensions were transferred to test tubes and centrifuged at $600 \times g$ for 5 min to collect the cells. To extract the formazan, 3 ml of *iso*-propyl alcohol was added to each tube, and the tubes were agitated thoroughly. After cell debris was removed by centrifugation at $600 \times g$ for 5 min, absorbance of the extracts was measured at 570 nm by Hitachi U-2000 spectrophotometer.

RESULTS

Effect of AL-toxin I on tomato calli

Calli of cvs. Mie-First, Earlypak 7 and Ace were cultured for 3 to 6 days on MS solid medium added with AL-toxin I at 0.05, 0.5 and 5 μg/ml. The growth of calli of susceptible cvs. Mie-First and Earlypak 7 was inhibited by the toxin at all concentrations within 3 days of incubation, and these calli showed browning. Calli of resistant cv. Ace, however, did not respond to the toxin even at 5 μg/ml and 6 days of incubation (Fig. 1).

Establishment of MTT-colorimetric assay

Dark-blue MTT-formazan produced by functionally active mitochondria has a maximum absorption at 570 nm[16]. The amount of the formazan produced by cultured cells could be determined precisely by measuring the optical density at 570 nm of test solutions, because filtrates of the cell culture did not show any absorption at this wavelength.

To determine the optimal condition for this assay, incubation time and temperature, and MTT concentration were examined. A relationship between incubation time and formazan production is shown in Fig. 2. Since formazan production by suspension-cultured cells of tomato cv. Mie-First reached a plateau after incubation for 6 hr, the incubation period for 6 hr was employed for subsequent experiments. Formazan production at various incubation temperatures (25, 30, 33 and 37°C) was also examined. After 6 hr incubation, the amount of formazan produced at 25, 30 and 33°C was 4.3, 17.5 and 33.5% of that at 37°C, respectively. The result indicates that the formazan production is temperature-dependent, and highest at 37°C. Cultured cells were also incubated for 6 hr at 37°C with various concentrations of MTT to determine the optimal MTT concentration. Formazan production was increased with increasing concentration of MTT, and reached a plateau at concentrations above 500 μg/ml (Fig. 2).

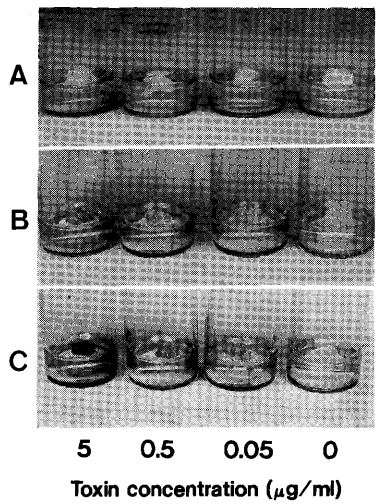

Fig. 1. Responses to AL-toxin I of calli from resistant cv. Ace (A), and susceptible cvs. Mie-First (B) and Earlypak 7 (C). Each of calli was incubated for 6 days on MS medium containing the toxin at concentrations of 0, 0.05, 0.5 and 5 μg/ml.

Finally, the relationship between the number of living cells and the amount of formazan generated was determined. The number of living cells of cv. Mie-First was determined by Evans blue staining. Absorbance at 570 nm was completely dependent on the number of living cells (Fig. 3). This linear correlation was observed even at a small number of cells (2,500 cells/well). On the other hands, when the cultured cells were pretreated in water at 100°C for 5 min, for-

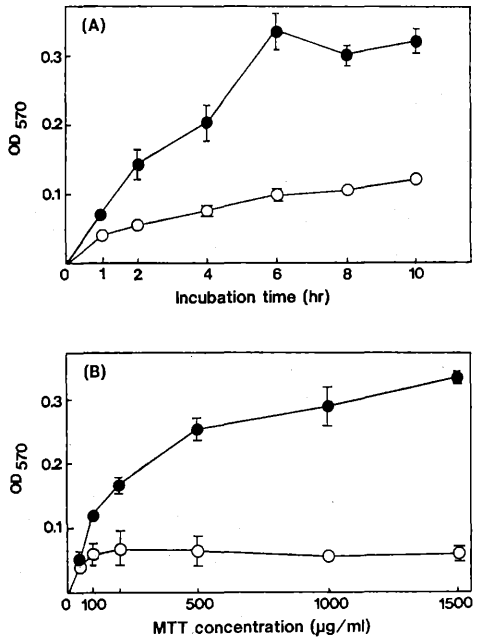

Fig. 2. Effects of incubation time (A) and MTT concentration (B) on amount of formazan produced by suspension-cultured cells from cv. Mie-First. (A) Cells were incubated at 37°C in a 24-well tray with 500 μg/ml MTT solution. (B) Cells were incubated at 37°C for 6 hr in a 24-well tray with MTT solution. ●: 10,000 cells/well, ○: 2,500 cells/well. The vertical bars represent the standard deviations of three experiments.

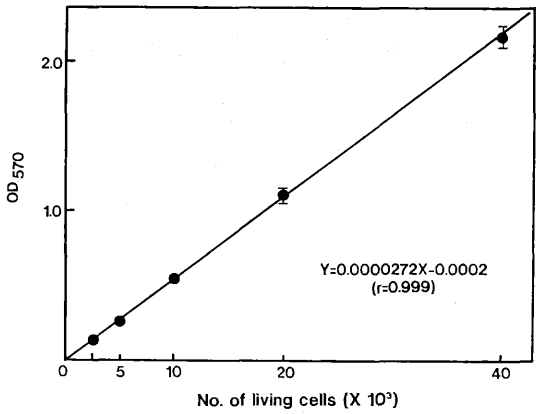

Fig. 3. Living cell-dependent production of formazan in MTT assay. Suspension-cultured cells (cv. Mie-First) were incubated at 37°C for 6 hr in 500 μg/ml MTT solution. The number of living cells was determined by Evans blue staining. The vertical bars represent the standard deviations of three experiments.

mazan production by the cells was not detected. The standard condition for MTT assay was thus determined to be 6 hr of incubation at 37°C with 500 μg/ml MTT.

Toxic activity of AL-toxin I on cultured cells determined by MTT colorimetric assay

The suspension-cultured cells of susceptible cv. Mie-First were incubated with various concentrations of AL-toxin I. Figure 4 shows the viability of the toxin-treated cells compared with the control cells incubated without toxin. Viability of cells decreased to 34% and 78% at concentrations of 1 and 0.1 μg/ml of the toxin, respectively, after the treatment for 3 days. A decrease in the viability of the toxin-treated cells became more significant as incubation time was prolonged. The toxin at 0.01 μg/ml, however, exerted little effect on the viability. Toxicity of the toxin to susceptible cultured cells was almost the same as that determined to cause

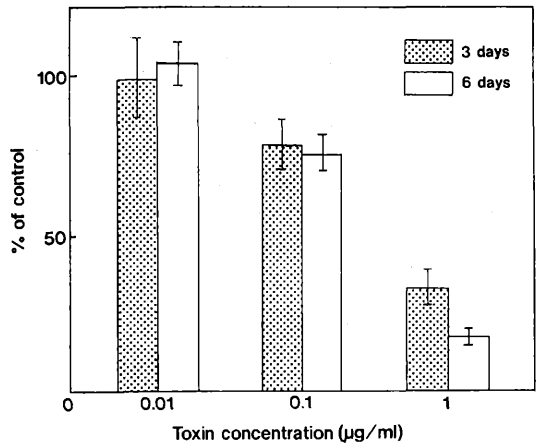

Fig. 4. Viability of suspension-cultured cells (10,000 cells/ml) from cv. Mie-First treated with various concentrations of AL-toxin I as assessed by MTT reduction after 3 and 6 days of incubation. Cell viability was expressed as the percentage of control incubated for 3 and 6 days without toxin treatment. The vertical bars represent the standard deviations of three experiments.

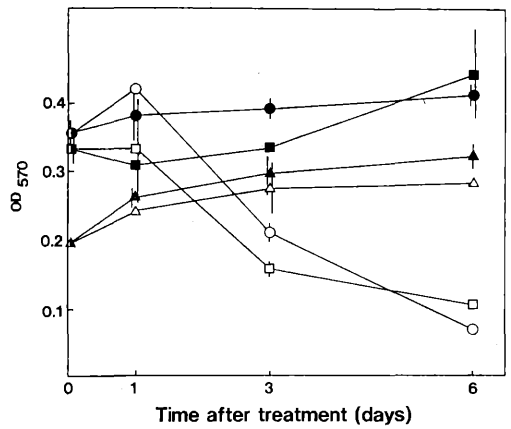

Fig. 5. Effect of AL-toxin I (5 μg/ml) on viabiltiy of suspension-cultured cells (10,000 cells/ml) from susceptible (S) and resistant (R) tomato cultivars. △: Ace (R)+Toxin, ▲: Ace (R), ○: Mie-First (S)+Toxin, ●: Mie-First (S), □: Earlypak 7 (S)+Toxin, ■: Earlypak 7 (S). The vertical bars represent the standard deviations of three experiments.

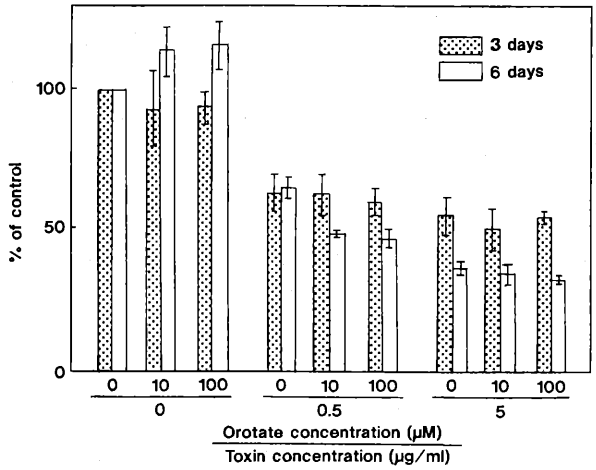

Fig. 6. AL-toxin sensitivity of suspension-cultured cells treated with orotate. Cells (10,000 cells/ml) of susceptible cv. Mie-First were incubated with mixtures of the toxin and orotate. Cell viability was determined by MTT assay 3 and 6 days after the treatment, and expressed as the percentage of control incubated for 3 and 6 days without toxin and orotate treatments. The vertical bars represent the standard deviations of three experiments.

necrosis on leaves by the toxin dilution end point method.

Difference in reactions to AL-toxin between cultured cells from susceptible and resistant cultivars

A decrease in formazan production by susceptible cells treated with the toxin became evident 3 days after exposure to the toxin (Fig. 5). In contrast, resistant cells were not affected by the toxin (5 μg/ml).

Effect of orotate on toxin respose of cultured cells

Effect of orotate on the toxicity of AL-toxin to cultured cells was reexamined by using MTT-colorimetric assay. Susceptible cells were incubated with mixture of the toxin (0.5 and 5 μg/ml) and orotate (10 and 100 μM). Orotate did not show any protection of cells from the toxin action at the high and low concentrations within 6 days of incubation (Fig. 6).

DISCUSSION

Bioassay methods, that are based on measurement of the weight or volume of cells as an indicator of proliferation and the plating efficiency of cells as a survival indicator, have been employed for the quantitative determination of toxin effects on plant cultured cells[4,24]. They, however, require a large amount of cells and a long peirod for the assay. Vital staining assays with FDA and Evans blue have also been used for detecting viable cells[5,23]. The staining methods have some difficulties in handling a large number of test samples and applying to cell aggregates and clumps, although they can be rapidly and simply performed with small quantities of cells. On the other hand, colorimetric assays using tetrazolium salts such as MTT[16] and TTC[22] seemed to be useful for rapid and quantitative determination of cell viability even with a large number of samples. Hence, we have established a standard protocol for measuring viability of plant cultured cell preparations by MTT colorimetry. The MTT method was proved to be an efficient and reliable one as demonstrated by the assay of AL-toxin effect on tomato cells: different responses to the toxin were observed between susceptible and resistant tomato cultivars, and toxin sensitivity of susceptible cultured cells was comparable with that of the leaf tissues. Furthermore, the MTT assay was found to be available to examine the effects of host-

selective toxins, that have different primary target organelles (AK-toxin, plasma membranes; AM-toxin, plasma membranes and chloroplasts; and ACR-toxin, mitochondria)[13], on cultured cells of each host plant (unpublished data).

Selective sensitivity to AL-toxin of tomato cultivars was remained detectable at the levels of callus and suspension-cultured cells as shown in Figs. 1 and 5, in contrast with previous studies by Fuson and Pratt[4] and Witsenboer *et al.*[24], who reported that there was no difference in toxin sensitivity of cultured cells between susceptible and resistant cultivars. Although the reason for this discrepancy has not been made clear, one possible explanation is that it is due to different bioassay system and tomato cell lines employed in these experiments. Somaclonal variation during *in vitro* cell culture might be another explanation for the disagreement[3].

On the other hand, apparent host-selective reactions in cell cultures from susceptible and resistant cultivars of crop plants have been reported as for AT-toxin[10] and HMT-toxin[6]. Furthermore, successful *in vitro* selections of disease-resistant genotypes by using these host-selective toxins have also been reported[6,15,19]. Application of AL-toxin to the selection of stem canker-resistant tomato cells and plants is a subject of further study.

In this study, FDA and Evans blue staining methods were used to examine the viability of toxin-treated cultured cells, but we often encountered difficulties in discriminating vital cells from dead cells even 6 days after toxin treatment (data not shown). Viability determination by these staining methods basically depends on the permeability of plasma membranes and on the esterase activity in cells, while MTT assay is based on the mitochondrial enzyme activity in living cells. The accuracy of MTT assay may probably lead to quantitative explanation of the AL-toxin-induced ultrastructural damages of mitochondria in susceptible leaf cells[18]. On the other hand, the abilities to produce formazan in cultured cells of susceptible tomato were not completely inhibited by AL-toxin even after incubation for 6 days, although the dead cells by heat treatment did not produce formazan. Further investigation is necessary on AL-toxin action to cultured cells.

We were unable to find any supporting evidence for the protective effect of orotate on AL-toxin actions reported by Gilchrist[7]. Our results were rather compatible with those described by Fuson and Pratt[4].

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和 文 摘 要

児玉基一朗・吉田拓司・尾谷 浩・甲元啓介・故 西村正暘：MTT 比色定量法を用いたトマト培養細胞に対する AL 毒素作用の検定

トマト・アルターナリア茎枯病菌が生成する宿主特異的毒素（AL 毒素）の培養細胞に対する作用について検討した。感受性および抵抗性トマト品種のカルスを毒素含有固形 MS 培地上で培養したところ，培養3日後で感受性品種カルスのみに生育阻害および褐変が誘起された。そこで，MTT 比色定量法を確立し，それを用いて毒素の培養細胞に対する効果を検討した。トマト懸濁培養細胞の場合，生細胞数と MTT 染色性の間にはきわめて高い相関が認められ，本法を用いて毒素の細胞毒性を定量的に評価することが可能であると思われた。感受性品種の培養細胞に対する毒素の効果は，葉に壊死を誘起する最小濃度にほぼ等しい濃度まで認められた。一方，抵抗性品種細胞の場合には，毒素の影響は認められなかった。以上の結果より，AL 毒素の選択的毒性は培養細胞レベルにおいても発現されるものと考えた。