# イチゴ黒斑病菌 (Alternaria alternata strawberry pathotype)の生成する宿主特異的毒素(AF毒素)に関する研究(5)


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# Studies on Host-Specific AF-toxins Produced by *Alternaria alternata* Strawberry Pathotype Causing Alternaria Black Spot of Strawberry (5) Effect of Toxins on Membrane Potential of Susceptible Plants as Assessed by Electrophysiological Method*

Fumio NAMIKI**, Hisashi OKAMOTO***, Kiyoshi KATOU***,
Mikihiro YAMAMOTO****, Syoyo NISHIMURA**, Shin-ichi NAKATSUKA**,
Toshio GOTO**, Keisuke KOHMOTO*****, Hiroshi OTANI*****,
and Anton NOVACKY******

**Abstract**

Plasma membrane dysfunction of strawberry and Japanese pear petiole cells treated with AF-toxins was studied by means of electrophysiological methods. The light-dependent and -independent components of membrane potential of susceptible strawberry were rapidly depolarized after AF-toxin I ($3.2\times10^{-6}$ M) treatment. AF-toxin II ($2.6\times10^{-6}$ M) did not cause any change in the membrane potential and its two components of susceptible strawberry. Resistant strawberry has shown no depolarization by AF-tioxin I ($3.2\times10^{-6}$ M) treatment. The membrane potentials between xylem vessel and parenchyma symplast of strawberry and Japanese pear petioles were measured by the xylem perfusion method. AF-toxin I induced inhibition of the activity of electrogenic ion pump on both susceptible strawberry and Japanese pear, but AF-toxin II induced it only in susceptible pear. In each case, respiration-dependent membrane potential difference rapidly decreased. These two AF-toxins did not cause any change in membrane potential of strawberry and Japanese pear resistant to the disease. Prior treatment of susceptible strawberry tissuse with AF-toxin II, non-toxic to strawberry, protected them from AF-toxin I action as was evident in electrophysiological indices. Respiration-dependent component of membrane potential in protected tissues was not influenced for at least 2 hr after AF-toxin I treatment.

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**Key words:** strawberry, AF-toxin, protective effect, membrane potential, active component, ATPase.

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** Faculty of Agriculture, Nagoya University, Nagoya 464, Japan 名古屋大学農学部
*** Faculty of Science, Nagoya University, Nagoya 464, Japan 名古屋大学理学部
**** Plantech Research Institute, Yokohama, Kanagawa, 227, Japan 植物工学研究所
***** Faculty of Agriculture, Tottori University, Tottori 680, Japan 鳥取大学農学部
****** Department of Plant Pathology, University of Missouri, Columbia, Missouri 65211, U. S. A. 米国，ミズリー大学

## Introduction

AF-toxins are capable of disturbing the membrane integrity only in the susceptible plants. The toxins caused an instantaneous and marked increase in loss of $K^+$ from the leaf tissues[4]. The increase in $K^+$ efflux was detectable within a few min after toxin treatment. Such toxin-induced dysfunctions of plasma membrane were also confirmed in the electron microscopy pictures which revealed that many invaginations of the membranes occurred within 1 to 3 hr after toxin treatment[13]. These earlier observations suggested that electrophysiological investigations at cellular level might provide more precise information concerning AF-toxin action on the membrane, and give some clues concerning the nature of disease susceptibility. Whether AF-toxins damage the host plasma membranes directly, or indirectly through affecting some metabolic processes has not been determined.

AF-toxins were found to consist of three phytotoxic molecular species (AF-toxins I, II and III) which possess apparently different host-selective toxicities[4,9]. Recently, the toxins have been structurally characterized[6,7] and pure preparations became available now for biological studies.

In this paper, we have measured electrophysiologically the changes of membrane potential differences in toxin-treated petiole cells of strawberry and Japanese pear. The objectives of this study are (1) to identify plasma membrane as possible target organelles carrying the primary action site for AF-toxin, (2) to determine whether the toxins inhibit the active components of membrane potential or passive ones, and (3) to re-examine the protective effect of pre-treatment with AF-toxin II on AF-toxin I-induced toxic action[9], by means of electrophysiological methods. Brief reports of some of this work have been published in a brief form[8,12].

## Materials and Methods

***Plants.*** Strawberry cultivars susceptible (Morioka-16) and resistant (Hoko-wase) to Alternaria black spot of strawberry were used in this study. Susceptible and resistant Japanese pear cultivars, Nijisseiki and Chojuro, respectively, were also used. Freshly harvested petioles of these plants were used throughout this study.

***AF-toxin preparalion.*** Toxins were purified by the method described previously[4,6]. Purified toxins were stocked in methanol ($10^{-4}$) at -20 C. The diluted toxin solutions were prepared by adding an adequate volume of perfusion solution to the stock methanol solution just before use.

As described earlier[4], AF-toxin I can induce veinal necrosis on leaves of both strawberry cv. Morioka-16 and Japanese pear cv. Nijisseiki. AF-toxin II is toxic to Nijisseiki pear, but not toxic to Morioka-16 strawberry. Conversely, AF-toxin III is highly toxic to Morioka-16 strawberry and slightly toxic to Nijisseiki pear. All these AF-toxins do not cause any visible toxicities on leaves of a number of resistant cvs. of strawberry and Japanese pear. AF-toxin III was not used in this study because of its ex-

tremely small yield from culture filtrates.

***Electrophysiological measurement.*** 1. *Determination of light-dependent component of membrane potential.* A petiole, 5 mm in length, was cut longitudinally into three slices. The middle slice, 2 mm in thickness and in width, was then mounted in a vertical 4 ml plexiglass chamber[10], which will be externally perfused with bathing solution (0.1 mM Na-MES buffer, 0.5 mM $CaSO_4$ and 50 mM mannitol) with or without AF-toxins. A glass micropipette (intracellular microelectrode), 0.5 $\mu$m in tip diameter, filled with 3 M KCl was inserted into mesophyll cell and another reference electrode into external solutions. The flow of the bathing solution was about 1 ml/min. Experiments were performed in a dark room. When necessary, the leaf sections were illuminated by a white incandescent lamp mounted in a microscope. The difference in membrane potential levels between dark and light phases were taken as "light-dependent component".

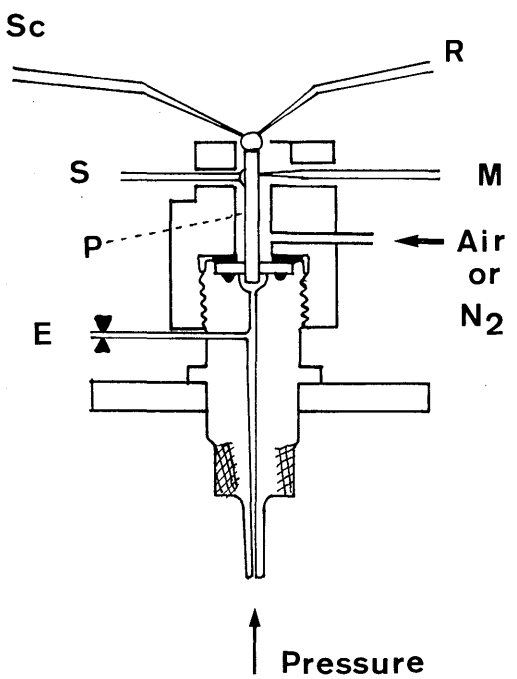


Fig. 1. Chamber for the pressurized intra-organ perfusion system. P, petiole segment 25 mm long, whose lower part near the cut end is glued to the hole of a flange with epoxy resin ; Sc, sucking pipette inserted into the droplet exuded from the upper cut end ; R, reference electrode ; M, intracellular glass microcapillary electrode ; S. surface electrode plugged with cotton thread and filled with 15 mM of KCl solution, which is in contact with a thin agar cake containing 15 mM of KCl that is placed on the surface of the segment ; and E, exaust.

2. *Determination of respiration-dependent component of membrane potential.* The basal part of a petiole segment (25 mm in length) excised from just below leaf blade was inserted into a hole (2.0-2.6 mm in diameter) bored in the center of a hard vinyl chloride flange and tightly fixed with compound epoxy-resin glue (Cemedine High Super, Cemedine Co., Tokyo). Small amount of silicon grease was smeared around the petiole just below the upper cut end of the segment in order to avoid the flowing-down of the exudate. Thereafter, the segment with a flange was tightly mounted by a screw divice into an acrylite chamber (Fig. 1). The air in the chamber could be replaced quickly by pure $N_2$ gas (99.998%). A perfusion solution (1 mM K-malate buffer, 0.5 mM $CaSO_4$, 1% sucrose), with or without AF-toxin, was pressed into xylem vessel from the lower cut end of the petiole by a Hamilton syringe (capacity 250 $mm^3$) with an appropriate load (10-20 g weight). Excess exudate of the perfusion solution appearing on the upper end was sucked off by a pipette connected to a pump. Details of the "xylem perfusion method" was described else-

where[2,5,11]. An intracellular microelectrode previously described[11] was inserted horizontally through a hole into parenchyma cells for the measurement of membrane potential difference between xylem vessel and parenchyma symplast. In this case, the reference electrode was placed in the xylem exudate on the upper cut end. The difference in membrane potential levels between air and anoxic phases in the dark were taken as "respiration-dependent component".

In both cases, membrane potentials were led via a pair of Ag/AgCl unpolarizable electrode to the non-inverting terminal of an operational amplifier (AM 405-2, Datal Systems Inc., Mansfield) of unity gain whose output was recorded on a chart recorder.

## Results

### *Effect of AF-toxins on light-dependent or light-independent components of membrane potential in strawberry petiole cells*

Petiole sections of susceptible strawberry (cv. Morioka-16) were bathed in perfusion solution. After a microelectrode was inserted into a single cell and the potential recording became stable in the light, the light was turned off. The difference in membrane potential levels between dark and light phases was taken as "light-dependent component". The light-dependent component of membrane potential was abolished in the dark, but membrane potential was recovered in the light to the original level. When AF-toxin I ($3.2\times10^{-6}$ M) was applied in perfusion solution in the dark, the membrane potential rapidly deporalized immediately after AF-toxin I reached the tissues. The light-dependent component of membrane potential was completely abolished by toxin application in the dark, and light-independent component was partially abolished. The membrane potential never recovered to the original level observed in toxin-free solution (Fig. 2-A).

In petiole sections of resistant strawberry (cv. Hoko-wase), no depolarization was observed by AF-toxin I ($3.2\times10^{-6}$ M) treatment in the dark, but both the membrane potential and its light-dependent component gradually as time passed (Fig. 2-B).

Application with AF-toxin II ($3.2\times10^{-6}$ M) in the dark did not cause any change in the membrane potential and in its two components in petiole sections of susceptible cv. Morioka-16 (Fig. 2-C).

### *Effect of AF-toxins on active or passive component of membrane potential in strawberry and Japanese pear petiole tissues*

A petiole segment of susceptible strawberry was mounted into an acrylite chamber, and the perfusion solution was passed into xylem vessel from the lower cut end of the petiole for 4 hr. An intracellular microelectrode was inserted horizontally. After membrane potential level became stable in the air phase, the air in the chamber was replaced quickly by $N_2$ gas. Under an anoxic condition, the cell membrane of petiole segment was depolarized immediately (Fig. 3). When $N_2$ gas was replaced by air, the cell membrane was repolarized. The differences in membrane potential levels between air and anoxic phases in the dark were taken as "respiration-dependent component" or simply "active component". When AF-toxin I ($3.2\times10^{-6}$ M) was applied in the perfusion so-

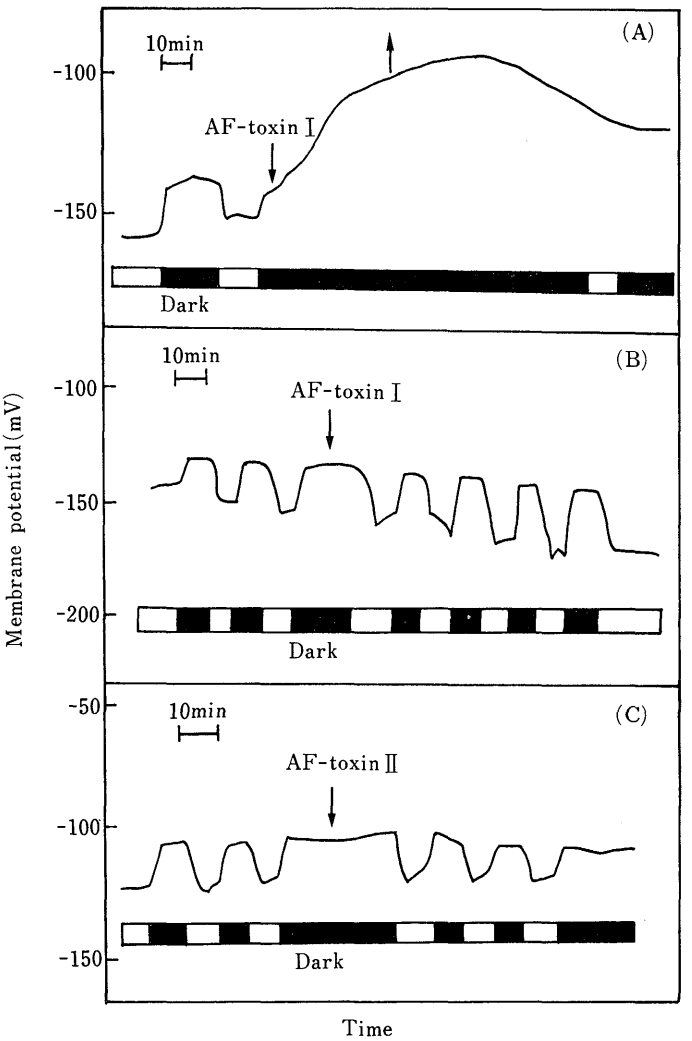


Fig. 2. Effect of AF-toxins on the membrane potential of light-dependent and -independent components of susceptible and resistant strawberry cells. Downward arrows indicate addition of toxin, and upward arrow indicates removal of toxin. Concentrations of AF-toxins I and II were $3.2\times10^{-6}$ and $2.6\times10^{-6}$ M. A : susceptible strawberry, B : resistant strawberry and C : susceptible strawberry.

lut'on under an aerobic phase, susceptible cell membrane immediately started to depolarize, and finally reached the potential level in anoxic phase. When $N_2$ gas was subsequently replaced by air, the active component was found to be decreased to one-fifth and was never recovered to the level observed before AF-toxin I application (Fig. 3-A). The cell membrane of resistant strawberry was not affected by AF-toxin I ($3.2\times10^{-6}$ M, Fig. 3-B and 4) and AF-toxin II (data not shown). Neither membrane potential nor its two components was affected by AF-toxin II, as was observed in the petiole segments of disease-susceptible strawberry exposed to AF-toxin II ($2.6\times10^{-6}$ M) under an aerobic condition (Fig. 3-C).

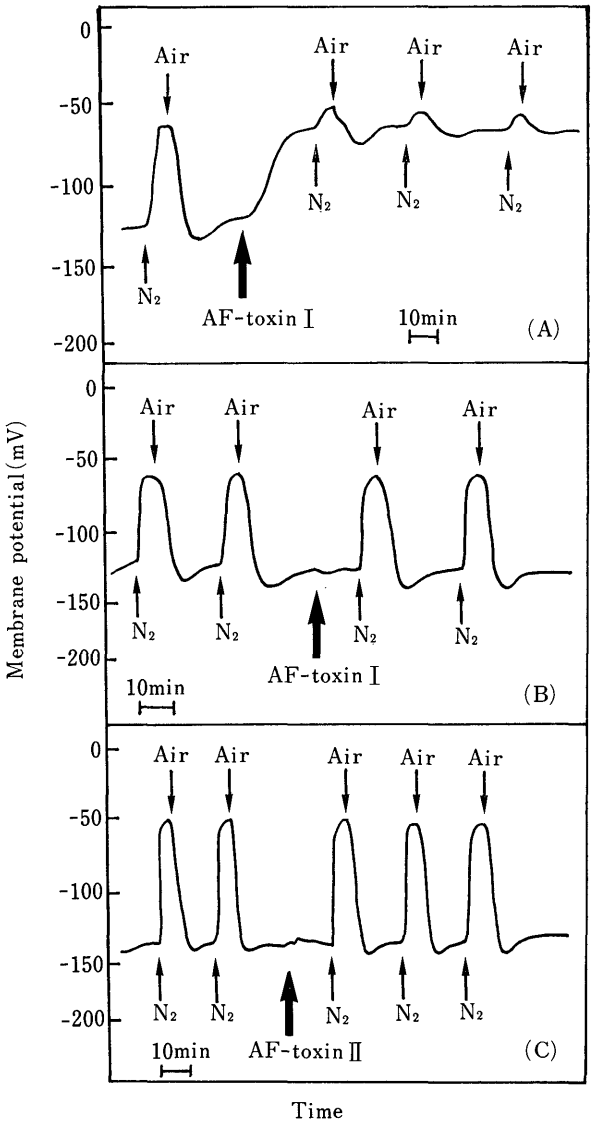


Fig. 3. Effect of AF-toxins on the respiration-dependent and passive components of membrane potential of susceptible and resistant strawberry cells. Upward arrows indicate replacement of air by $N_2$, and downward arrows indicate re-aeration. Thicker arrows indicate addition of toxin to perfusion solution. Concentrations of AF-toxins I and II were $3.2\times10^{-6}$ M and $2.6\times10^{-6}$ M. A : susceptible strawberry, B : resistant strawberry and C : susceptible strawberry.

The membrane potential of susceptible Japanese pear petioles was depolarized about 30 min after AF-toxin II ($2.6\times10^{-6}$ M) application (Fig. 5), indicating that a certain period of time is required for toxin molecules to reach the cell membrane. AF-toxin I also caused the depolarization of cell membrane of susceptible Japanese pear (data not shown). Resistant strawberry was not influenced by AF-toxin I ($3.2\times10^{-6}$ M) and II ($2.6\times10^{-6}$ M).

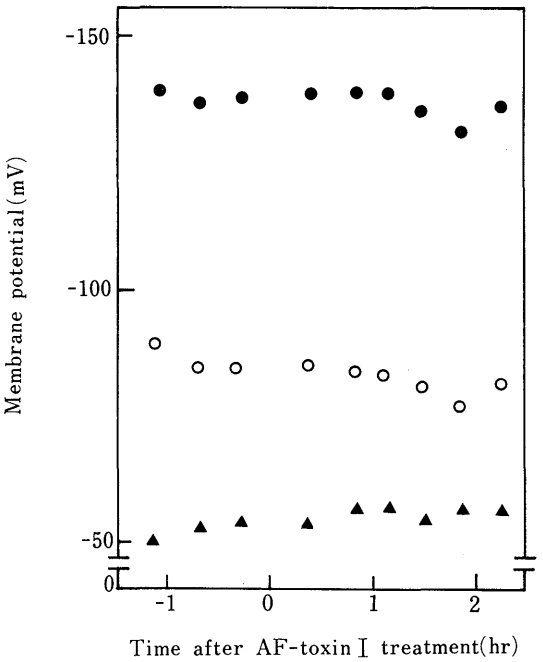


Fig. 4. Effect of AF-toxin I ($3.2\times10^{-6}$M) on the membrane potential (●) and its two components (active, ○ and passive, ▲) of resistant strawberry cells. Toxin was added to the perfusion solution at time 0.

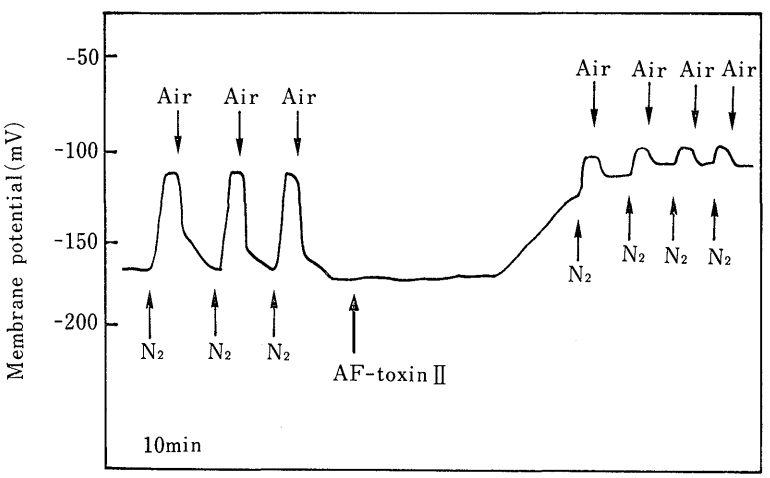


Fig. 5. Effect of AF-toxin II ($2.6\times10^{-6}$M) on the membrane potential of susceptible Japanese pear cells. Upward arrows indicate replacement of air by $N_2$, and downward arrows indicate re-aeration. Thicker arrow indicates addition of toxin to perfusion solution.

***Protective effect of pre-incubation with AF-toxin II on the toxic action of AF-toxin I***

On the basis of assay of leakage of potassium from the tissues and induction of veinal

necrosis, we demonstrated that AF-toxin II protects strawberry leaves from AF-toxin I-induced damages[9]. By means of electrophysiological methods, the toxic action of AF-toxin I to strawberry was re-examined.

The basal part of a petiole segment of susceptible strawberry was soaked in AF-toxin II ($2.6\times10^{-5}$ M) solution for 20 hr. The segment was mounted into an acrylite chamber, and the perfusion solution containing AF-toxin II ($2.6\times10^{-5}$ M) was pressed into xylem vessel from the lower cut end of the petiole for 4 hr. Then, a glass microelectrode was inserted. After membrane potential level became stable in the air phase, the air in the chamber was replaced quickly by $N_2$ gas. Under an anoxic condition, the membrane potential was depolarized immediately. Thus, the cell membrane of susceptible petiole was not influenced even at high concentration of AF-toxin II ($2.6\times10^{-5}$ M). AF-toxin I ($3.2\times10^{-6}$ M), which is sufficient to cause the depolarization of suceptible cells, was added to perfusion solution under an aerobic phase. Interestingly, the membrane potential and its active and passive components of susceptible cell were almost constant at least for 2 hr after AF-toxin I application, although, as time passed, these levels gradually decreased (Fig. 6).

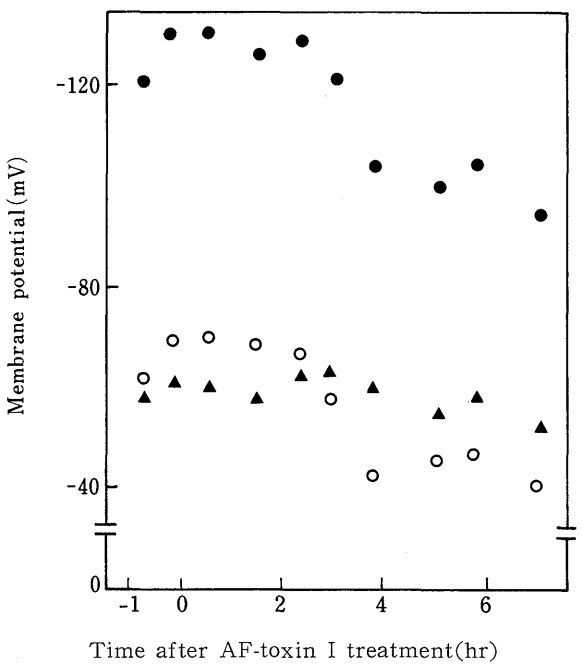


Fig. 6. Effect of pre-incubation with AF-toxin II ($2.6\times10^{-5}$M) on the action of AF-toxin I ($3.2\times10^{-6}$M) on the membrane potential (●) and its two components (passive, ▲ ; active, ○) of susceptible strawberry cells. Materials were pre-incubated with AF-toxin II for 24 hrs and AF-toxin I was added to the perfusion solution at time 0.

## Discussion

AF-toxins depolarized the membrane of susceptible plant cells shortly after toxin treatment. The depolarization resulted from the loss of active component of membrane potential, suggesting that the electrogenic $H^+$-pump might be selectively damaged. The electrophysiological results recorded here were in harmony with previous observations on biological activities (*e. g.* leaf veinal necrosis and $K^+$ efflux from leaf tissues) of each AF-toxin on susceptible plants[9] ; AF-toxin I induced depolarization both in susceptible strawberry and Japanese pear, whereas AF-toxin II induced it only in susceptible Japanese pear. Both toxins, however, did not cause any effect in any of the experiments with the strawberry cv. Morioka-16 and Japanese pear cv. Chojuro, immune to the disease.

An analogous depolarization of plasma membrane has recently been found in the combination of sugarcane tissues and HS-toxin produced by *Helminthosporium sacchari*[14]. However, the patterns observed in the present work were somewhat different. The loss of active component of membrane potential in sugarcane tissues after HS-toxin treatment could be recovered by washing the leaf sections with a toxin-free solution, and at low concentrations of HS-toxin the depolarized membrane potential partially recovered. In our combination of host and toxins, however, the light-dependent and-independent components of membrane potential were markedly decreased by toxins, and the depolarized potential was not recovered to the original level by washing with a toxin-free solution. Such rapid and irreversible action of AF-toxins is probably due to a direct and strong effect on electrogenic $H^+$-pump on plasma membrane. If this was the case, it would follow that effects of AF-toxins on the isolated plasma membrane or purified membrane ATPase was similar.

In earlier paper[9], we reported that the appearance of visible symptoms and physiological damages caused by AF-toxin I in susceptible strawberry tissues are partially inhibited by pretreatment with AF-toxin II. Results of electrophysiological experiments about this problem were somewhat similar to the previous results with whole tissues. Thus, it seems possible that nontoxic AF-toxin II combines with a certain site on plasma membrane of host plant, protecting it from subsequent attack by AF-toxin I.

### Literature cited

1. Duvick, J. P., Daly, J. M., Klatky, Z., Macko, V., Acklin, W. and Arigoni, D. (1984). Plant physiol. 74 : 117-122.
2. Katou, K. and Okamoto, H. (1985). Kagaku 55 : 560-568.
3. Livingston, R. S. and Scheffer, R. P. (1984). Physiol. Pl. Path. 24 : 133-142.
4. Maekawa, N., Yamamoto, M., Nishimura, S., Kohmoto, K., Kuwata, M. and Watanabe, Y. (1984). Ann. Phytopath. Soc. Japan 50 : 600-609.
5. Mizuno, A., Kojima, H., Katou, K. and Okamoto, H. (1985). Plant, Cell and Environ. 8 : 525-529.
6. Nakatsuka, S., Ueda, K., Goto, T., Yamamoto, M., Nishimura, S. and Kohmoto, K. (1985) Tetrahedron Lett. 27 : 2753-2756.
7. Nakatsuka, S., Ueda, k., Goto, T., Yamamoto, M., Nishimura, S. and Kohmoto, K. (1985). Pres-

ented at The Third International Kyoto Conference on New Aspects of Organic Chemistry (Kyoto, Japan) (Abstr.).
8. Namiki, F., Okamoto, H., Katou, K., Yamamoto, M., Nishimura, S., Kohmoto, K. and Otani, H. (1986). Ann. Phytopath. Soc. Japan 52 : 133.
9. Namiki, F., Yamamoto, M., Nishimura, S., Nakatsuka, S., Goto, T., Kohmoto, K. and Otani, H. (1986). Ann. Phytopath. Soc. Japan 52 : 428-436.
10. Novacky, A., Karr, A. L. ana Van Sambeek, J. W. (1976). Bioscience 26 : 499-505.
11. Okamoto, H., Mizuno, A., Katou, K., Ono, Y., Matsumura, Y. and Kojima, H. (1984). Plant, Cell and Environ. 7 : 139-147.
12. Okamoto, H., Namiki, F., Nishimura, S. and Takai, S. (1985). The 50th Meeting of Bot. Soc. of Japan No. 2a-S-7 (Abstr.).
13. Park, P., Nishimura, S., Kohmoto, K., Otani. and Scheffer, R. P. (1974). Ann. Phytopath. Soc. Japan. 47 : 488-500.
14. Schröter, H., Novacky, A. and Macko, V. (1985). Physiol. Pl. Path. 26 : 165-174.
15. Yamamoto, M., Nishimura, S., Kohmoto, K. and Otani, H. (1984). Ann. Phytopath. Soc. Japan 50 : 610-619.

## 和 文 摘 要

並木史郎・岡本　尚・加藤　潔・山本幹博・西村正暘・中塚進一・後藤俊夫・甲元啓介・尾谷　浩・Anton Novacky：イチゴ黒斑病菌（*Alternaria alternata* strawberry pathotype）の生成する宿主特異的毒素（AF毒素）に関する研究　(5) 宿主細胞膜に対する AF 毒素の作用の電気生理学的解析

電気生理学的方法を用いて，AF 毒素を処理したイチゴおよびナシ葉柄細胞の膜機能の変化を調査した。また，感受性イチゴへの毒素Ⅰの作用に対する毒素Ⅱの保護効果について再検討した。AF 毒素Ⅰは $3.2\times10^{-6}$ M という低濃度で感受性イチゴの光依存性および非依存性の膜電位成分を急激に減少させた。AF 毒素Ⅱは感受性イチゴに何らの作用もおよぼさなかった。なお，抵抗性イチゴはいずれの毒素にも反応しなかった。次に，イチゴおよびナシ葉柄を用いて道管灌流法によって道管―生細胞間の界面膜電位差を測定した。毒素Ⅰはイチゴとナシの，毒素Ⅱはナシの，それぞれ感受性品種のみに起電性イオンポンプ活性の阻害をひきおこし，呼吸依存性の膜電位差が急激に減少した。また，両毒素とも抵抗性品種には何ら作用をおよぼさなかった。感受性イチゴにあらかじめ毒素Ⅱを24時間処理してから毒素Ⅰを灌流すると，初期において呼吸依存性の膜電位差の急激な減少はみられなかった。以上の結果から，AF 毒素の初期作用は感受性植物の原形質膜の機能喪失であり，毒素Ⅱはイチゴに対して毒素Ⅰによる原形質膜への初期作用を著しく遅延させることがわかった。