

Alternaria

alternata群病原菌における宿主特異的毒素生成の制御

高温度ストレスによる孢子発芽時の毒素生成の阻害

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	50巻2号
巻号補足	
掲載ページ	p. 189-196
発行年月	1984年4月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター

Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Metabolic Regulation of Host-Specific Toxin Production in *Alternaria alternata* Pathogen (1). Suppression of Toxin Production from Germinating Spores under High Temperature Stress*

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柘植尚志**・西村正暘** : *Alternaria alternata* 群病原菌における宿主特異的毒素生成の制御 (1). 高温度ストレスによる孢子発芽時の毒素生成の阻害*

Abstract

The effect of thermal treatment on the pathogenicity of spores of *Alternaria alternata* Japanese pear pathotype was examined. When the virulent spores were subjected to a thermal treatment (*e. g.*, at 50°C for 10 sec in water), they were not significantly inhibited their abilities of spore germination, elongation of germ tube and appressorium formation. However, they remarkably lost their pathogenicity to Japanese pear leaves susceptible to the black spot disease as well as productivity of host-specific AK-toxin during spore germination. Such an acquired effect on the pathogenicity and the toxin productivity was lost within about 24 hr after the treatment. When the air-dried viable spores were continuously exposed to hot summer temperatures for 13 days in field, the spores lost their pathogenicity and AK-toxin productivity without any visible damages on their germination ability. The above data suggest that certain thermal treatment of the spores of *A. alternata* Japanese pear pathotype causes to reduce their pathogenicity through the loss of AK-toxin productivity.

(Received October 11, 1983)

Key Words: *Alternaria alternata* pathogen, host-specific toxin, high temperature stress, metabolic regulation.

Introduction

Previous research concerning host-specific toxins in a group of pathogenic *Alternaria alternata* seems warranted for explaining their significant role in pathogenesis^{3,4,5}. In most, if not all, of the toxins, there is now sufficiently quantitative evidence that toxins can be detected in the spore germination fluids of each virulent pathogen, but not in those of avirulent ones^{1,8}. This is a significant point, because such toxins released from germinating spores at the site of initial contact of the pathogen with host plant surface seem to lead to successful pathogenesis in plant tissues. If the toxin production is inhibited by some means during spore germination without any influence on other functions, then the virulence exhibited by the pathogen can conceptually be expected to be reduced.

* This work was supported in part by Research Grant No. 511012 (1979-1981) from the Ministry of Education, Science and Culture of Japan

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The first aim of this work was to determine whether or not brief exposure of spores to high temperature, prior to their germination, affects the production of "release toxin" from the germinating spores. In addition, we have attempted to examine the possibility of high temperature stress in changing virulent *A. alternata* pathogens toward lesser virulent ones through inhibitory effect of high temperature on toxin production during hot summer months in field. The present results with Japanese pear leaves and *A. alternata* Japanese pear pathotype (previously called *A. kikuchiana*) should be helpful in understanding the importance of host-specific toxin as an initiation factor of successful pathogenesis. Brief reports of this work have been published^{2,3,9)}.

Materials and Methods

Plant. Freshly harvested young leaves of Japanese pear cultivar (cv. Nijisseiki) susceptible to *A. alternata* Japanese pear pathotype (causal fungus of black spot disease) and to its host-specific toxin (AK-toxin) were used in all experiments.

Preparation of spores. Spores were collected by the following method: 20 days old mycelial mats of the virulent isolate No. 15A of *A. alternata* Japanese pear pathotype, which grew on Richards' medium, were separated from the culture medium, washed several times in running tap water, squeezed to remove excess moisture and then placed on filter papers (Toyo filter paper No. 2) in a chamber covered with moist filter paper. The chamber was incubated at 25 C for 2 days to obtain abundant sporulation on the mycelial mats. The mycelial mats, laden with spores, were flushed with distilled water and resulting spore suspensions were filtrated through two layers of guaze to remove debris. The suspension was then centrifuged at 1,500 rpm for 3 min by Kubota KN-70 to collect spores to be used.

Thermal treatment of spores. The effects of thermal treatment on pathogenicity of pathogenic spores were tested by holding spores in water for specified time periods at several different temperatures. Test tubes (ϕ 12 mm) containing 3 ml distilled water were stood in water bath kept at specified constant temperatures. One drop of highly concentrated spore suspension (about 1.5×10^8 spores ml^{-1}) was dropped in the test tubes. After the treatment, spore suspensions in test tubes were cooled quickly and were adjusted to give concentration of 10^6 spores ml^{-1} with distilled water. In the case of thermal treatment in air, air-dried viable spores were exposed to high temperatures for specified time periods, and then suspended in distilled water to give final concentration of 10^6 spores ml^{-1} .

Assay. To observe the infection behaviors of spores, the uniform suspension (10^6 spores ml^{-1}) of heat treated and untreated spore suspensions were placed on cellophane membrane (3×1 cm), prepared from Cellophane Tubing-Seamless for dialysis (Wako Pure Chemical Industries, Ltd.) and incubated in a moist chamber at 25 C. After various incubation times, the membranes were stained with lactophenol cotton blue, and spore germination, length of germ tube and appressorium formation were measured. For the pathogenicity test, freshly detached young leaves of susceptible Japanese pear were cut off into two parts along with their midribs. One part was inoculated with heat treated spores and the other part was inoculated with untreated spores as a control.

After incubation in a moist chamber at 25 C for 20 hr, the number of black necrotic spots appeared on the leaves were counted. In test for the release of Ak-toxin from germinating spores, spore suspension (10^6 spores ml^{-1}) was placed on paper towel in a chamber. After various times of incubation, the spore-germinating fluids were harvested by squeezing the towel and spores were removed by centrifugation (at 2,000 rpm for 5 min). The supernatants were then bioassayed for AK-toxin production. The test solutions were prepared by successive 2-fold dilution with distilled water, and one drop of each solution was placed on across scratch of susceptible pear leaves. The maximum dilution end point for toxin-induced veinal necrosis was determined.

Results

Effect of pre-treatment with heat on pathogenicity of virulent spores of A. alternata

Dormant spores of virulent isolate of *A. alternata* Japanese pear pathotype were held at 50 C in water for different time periods, then sprayed on susceptible pear leaves and

incubated at 25 C for 20 hr. The number of black necrotic spots and the rate of spore germination on the leaves were measured (Fig. 1). Spot formation with spores treated at 50 C for 10 sec was remarkably reduced in number as compared with that of untreated ones, while no significant decrease was observed in the rate of spore germination. The treatment at 50 C for 30 sec caused almost complete inhibition of pathogenicity expression, in spite of still remaining considerable degree of spore germination.

The temperature and the exposure time required for reducing pathogenicity expression were determined more precisely. Dormant spores were placed in water held at different temperatures ranging from 50 to 55 C for various times, cooled by immersing in water at about 5 C and then sprayed on pear leaves (Fig. 2). As a result, times required for significant reduction of pathogenicity expression with little effect on spore germination were 10–20, 5–10, 5–10 and 5 sec at 50, 51, 52

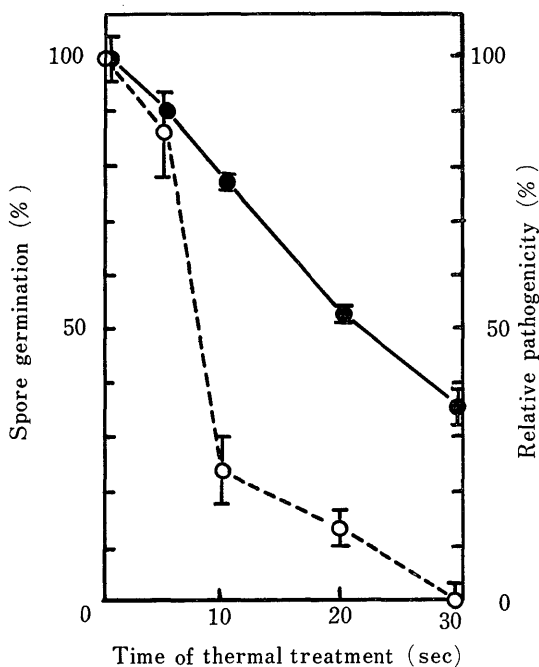


Fig. 1. Effects of thermal treatment (50 C, in water) on spore germination (●) and pathogenicity (○) of virulent spores of *A. alternata* Japanese pear pathotype. Relative pathogenicity represents the percentage of number of black necrotic spots on the half leaf inoculated with treated spores, to those on the other side inoculated with untreated spores. The vertical bars represent the standard errors.

and 53 C, respectively. At 54 and 55 C, however, the heat exposure times required for securing difference between reduction of pathogenicity expression and that of the ability of spore germination became subtle and impossible to determine.

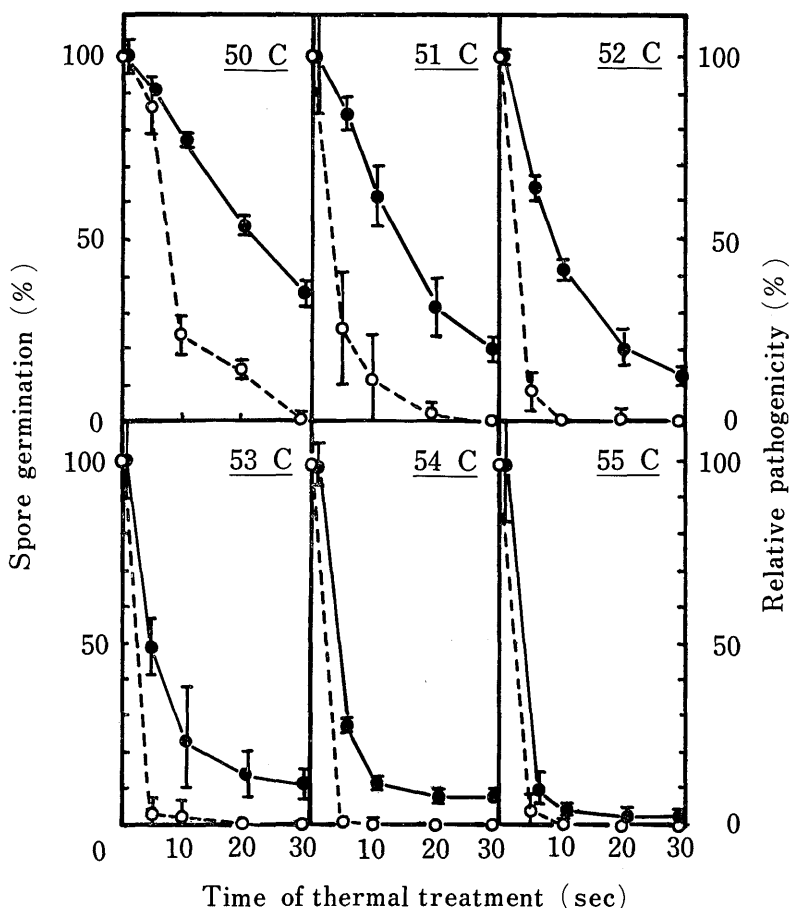


Fig. 2. Effects of thermal treatments at several temperatures (in water) on spore germination (●) and pathogenicity (○) of virulent spores. The vertical bars represent the standard errors.

Effect of pre-treatment with heat on AK-toxin productivity

The above data suggest that brief exposure of dormant spores to heat before germination leads to suppression of virulence through its inhibitory effect on toxin production. Heat dosage of 50 C-10 sec was employed for determining the heat effect on toxin production by germinating spores. Untreated spores were served as control (Fig. 3). After incubation on a cellophane membrane at 25 C for specified time periods, the rate of spore germination, the length of germ tube and the rate of appressorium formation were measured under a microscope. In another experiment, spore-germinating fluids were collected by centrifugation. The supernatants were subjected to bioassay for AK-toxin as given in Materials and Methods. Results showed that thermal treatment of spores caused suppression of toxin production by germinating spores without any sig-

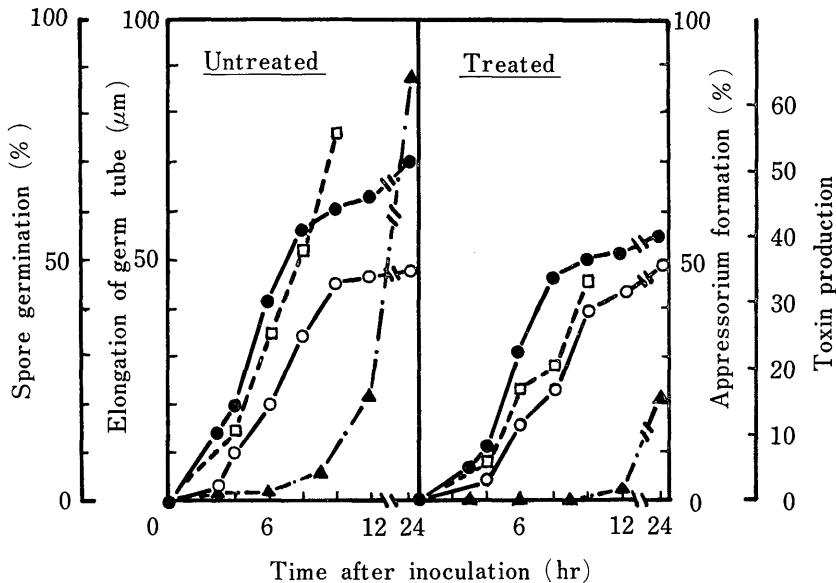


Fig. 3. Effects of thermal treatment (50 C-10 sec, in water) on spore germination (●), elongation of germ tube (□), appressorium formation (○) and toxin production (▲) of virulent spores. Appressorium formation represents the percentage of appressorium per germ tube. Toxin production represents the dilution end point for toxicity as assessed by veinal necrosis on leaf of susceptible Japanese pear cv. Nijisseiki.

nificant effect on several infection behaviors. Toxin production by the treated spores was first evident 12 hr after inoculation, whereas toxin production by untreated spores was evident 3 hr after inoculation.

Such a heat-induced inactivation of toxin productivity and virulence of spores was reversible and they were recovered almost completely when treated spore suspensions were incubated at 4 C for 24 hr after the treatment.

Effect of high temperature in air on pathogenicity

A mass of air-dried viable spores sporulated on mycelial mat of virulent *A. alternata* was placed on a paraffin paper in a plastic chamber (30×22×6 cm), located 30 cm above the ground in field and was exposed to hot summer temperatures during the period of 3 to 16 August in 1983. During the exposure period, abilities of the spores to germinate, to exhibit pathogenicity and to produce AK-toxin were measured under laboratory conditions (at 25 C) every day (Fig. 4A). With the passage of exposure time, both pathogenicity and AK-toxin productivity tended to reduce remarkably, while the ability to germinate was still preserved. Daily maximum temperatures in the experimental field were over 30 C from 3 to 15 August. Total time, for which the temperatures in field ranged over 30 C during the experiment, was 117 hr (Fig. 4B).

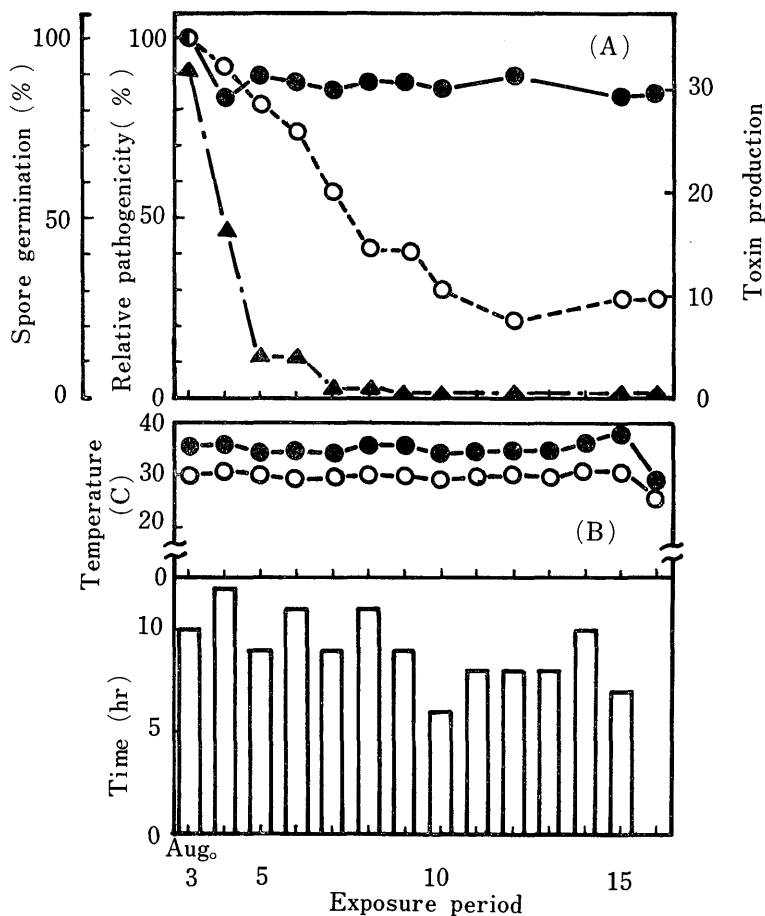


Fig. 4. (A) Changes of spore germination (●), pathogenicity (○) and toxin production (▲) of virulent spores exposed to high temperatures in field (August 3 to 16, 1983). (B) Daily maximum (●) and average (○) air temperatures and daily hours ranged over 30°C from August 3 to 16 in 1983.

Discussion

Otani *et al.* indicated that when Japanese pear leaves susceptible to the black spot disease were subjected to a heat shock (*e. g.*, at 50°C for 20 sec in water), they became almost completely insensitive to AK-toxin, but became more susceptible toxin-producing fungus (*A. alternata* Japanese pear pathotype). Such heat-shocked leaves also became susceptible even to toxinless mutant or nonpathogenic *A. alternata*^{6,7}. These results are helpful to understand the molecular basis of fungal infection as well as the heat-altered sensitivity of host tissues to the pathogens depending on host-specific toxin.

In the work presented here, however, thermal treatment was applied to pathogenic spores, by which we expected to reduce pathogenicity and/or toxin productivity without any significant effect on other infection behaviors. Such an attempt has not been de-

scribed previously. Result showed that the *A. alternata* pathogen depending on AK-toxin could not exhibit their specific virulence without "release toxin" from the germinating spores, and that heat shock (*e. g.*, at 50 C for 10 sec in water) of dormant spores before the germination affected the virulence through the inhibition of toxin production. Although these results must be interpreted with caution, they appeared to reflect a change in enzymes or more complex systems which are involved in toxin metabolism.

As was often discussed for some pathogens which produce host-specific toxins, the field isolates of *A. alternata* Japanese pear pathotype frequently lose the ability to produce AK-toxin when they are kept in culture. The mechanism is still far from understood. Considering this problem, however, in this paper the possible loss of pathogenicity under high mid-summer temperature in field was investigated. The results indicated that the spores kept in field became less virulent along with reduction of toxin production from germinating spores, although their ability to germinate was still remained. Field observations obviously indicate that the incidence of black spot disease of Japanese pear is most severe during spring and autumn seasons, and is limited during hot summer months, because high temperatures over 30 C are the major factor limiting the disease. Although such high temperatures contribute toward inhibiting the growth of pathogen as well as changing host tissues into toxin-insensitive^{6,7)}, our results indicated that the suppression of pathogenicity might also be of great significance in summer reduction of the disease.

In addition to thermal treatment, toxin metabolisms in germinating spores of pathogen are also regulated artificially by some chemicals with little effect on spore germination (unpublished). In both cases, metabolic inhibition of toxin production is now producing valid evidence for the importance of "release toxin" in pathogenesis.

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和 文 摘 要

Alternaria alternata 群病原菌における宿主特異的毒素生成の
制御 (1). 高温度ストレスによる孢子発芽時の毒素生成の阻害

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ナシ黒斑病菌の病原性発現に及ぼす高温度ストレスの効果について検討した。本菌の分生孢子を 50℃ の温湯中に10秒間浸漬すると、二十世紀ナシ葉に対する病原性発現が著しく阻害された。その際、孢子の発芽、発芽管の伸長および付着器の形成などは殆んど影響を受けなかった。しかし、孢子発芽時の宿主特異的毒素 (AK-毒素) の生成・放出は著しく抑制されることが観察された。なお、温湯処理した分生孢子は、処理後 4℃ 下に24時間放置することにより、AK-毒素生成能力を回復し、その結果、病原性発現能力も回復した。また、風乾した分生孢子を夏季 (1983年8月3日～8月16日) に野外に放置した結果、孢子の発芽能力は殆んど影響を受けることなく、孢子の発芽時の AK-毒素生成能力および病原性が著しく低下することが見出された。以上の結果から、ナシ黒斑病菌の分生孢子に対する高温度ストレスは、孢子発芽時の AK-毒素生成に対して阻害効果を示し、その結果、病原性の低下を引き起こすものと考えられ、本菌の病原性発現における孢子発芽時の AK-毒素の重要性が示唆された。