SOME ASPECTS OF EPIDEMIOLOGY OF *ALTERNARIA ALTERNATA* TOBACCO PATHOTYPE

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ABSTRACT
*Some fungi belonging to genus **Alternaria** are plant parasites that easily can change when exposed under different conditions. Variability of **Alternaria alternata** tobacco pathotype includes morphology, physiology, sporulation and pathogenicity of this fungus. Diversity in color of colonies appeared even in a single spore isolate used in this study. Numerous of spores were obtained when the pathogen was grown under near UV light at 25°C. The spores germinated on leaf surface in 24 hr in high humidity and infection hyphae of germinating spores penetrated epidermal cells directly.*

Introduction

Brown spot is a disease, caused by a fungus of genera *Alternaria* with considerable variation over its taxonomy name. The pathogen is known as *Alternaria tennuis* Nees, *Alternaria longipes* (El.& Ev.) and *Alternaria alternata* (Fries) Keissl. (Spurr and Main, 1974). *Alternaria alternata* tobacco pathotype is the name accepted for the causal pathogen of brown spot disease of tobacco in this article.
It has been established that isolates of *Alternaria* are genetically variable and any given mycelium may become heterocaryotic, because of the nature of the pathogen. Conidia produced from such mycelium may be dissimilar genetically. Differences between isolates of the pathogen are concealed through pronounced polymorphism of the conidia of individual isolate (Lucas, 1971). At the same time the conidial size, shape and segmentation varies considerably, depending on factors such as fungus strain, age of spores, substrate, pH, temperature, humidity, light (Lucas, 1975).
The pathogenicity of *Alternaria alternata* isolates is also quite unstable. Von Ramm and Lucas (1963) reported that non-sporulating sectors develop often in culture even under optimum condition of sporulation and virulent isolates soon become avirulent. Weekly subculturing on potato dextrose agar caused a rapid drop in pathogenicity (Lloyd, 1972). However, Spurr (1974) made 13 serial transfers on V-8 agar or PDA agar and found that the transfer did not alter the virulence of isolates.
Conidia of *Alternaria alternata* tobacco pathotype begin to germinate in the water film on leaf surfaces in less than one hour. Often more than one germ tube is produced, and they penetrate the leaf cells directly through walls or through stomates. Stomatal penetration appears to occur by chance and, hyphae sometimes grow over stomata without penetration. Although

brown spot legions may be visible within 2 to 8 days after inoculation, Norse (1971) detected the maximum observed incubation period of about 35 days before the appearance of the symptoms.
The data reported here present our experience with this pathogen, concerning some aspects of its morphology and epidemiology.

Materials and Methods

Pathogen. *Alternaria alternata* tobacco pathotype isolate 0-268 was kindly provided by Dr. Kohmoto from Faculty of Agriculture, Tottori University, Japan. Cultures were maintained on potato-dextrose agar (PDA) slants in test tubes at 4° C. The fungus was cultivated on PDA agar in petri dishes in 25°C in the dark for the experiments in this work.

Plant material. Three tobacco (*Nicotiana tabacum* L.) cultivars were used in this study. Two of them, Shiroenshu 1 and Shiroenshu 601 are Japanese tobacco cultivars that appeared to be susceptible and moderately resistant to the disease respectively. Tobacco cultivar Beinhart 100.1 is known as resistant to brown spot. All plants were grown in pots in the greenhouse and fertilized with the equal amount of fertilizer. Fully expanded leaves of approximately 40 cm high plants were used as a leaf material for detached leaf bioassays.

Conidial production. The possibility that isolate can lose its sporulation ability and difficulties with obtaining the spores sometimes prompted an evaluation of the sporulation conditions. For that purpose, petri dishes with mycelium grown on PDA for two weeks were divided in four groups. The mycelium mat grown on the surface of agar media was removed by brush under sterile conditions and the four groups were exposed separately under following conditions: continuous light, light period 16/8 hr light/darkness, continuous near UV light at 360 nm and continuous darkness. Four other groups prepared on the same way, were exposed under the same conditions, but they were kept at 4° C in darkness for 48 hr before the treatments to examine the role of low temperature and darkness for initiation of sporulation. Spores were brushed from petri dishes in distilled water and washed twice by centrifugation at 2000 rpm for 5 minutes. The pellet of spores was suspended in 10 ml of distilled water and the number of spores per milliliter was counted under a microscope.

Inoculum potential. To determine the effect of inoculum potential on brown spot development, tobacco leaves of susceptible cultivar Shiroenshu 1 were inoculated by different concentrations of spore suspension. Spore production was initiated in petri dishes with fully expanded mycelium maintained under near UV light. Spore suspensions of concentration 10^3, 5.10^3, 10^4, 2.10^4, 3.10^4 and 6.10^4 conidia per milliliter were prepared as described above. Drops of 10 µl of spore suspension were applied on upper side of tobacco leaves of susceptible cultivar Shiroenshu 1. The leaves were maintained at room temperature for several hours to allow the drops to dry, and they were kept on moist filter paper in petri dishes to give high humidity thereafter. The lesion expansion in different conidial concentrations was examined a week after the treatment.

Results and Discussion

Variability in *Alternaria alternata* tobacco pathotype. *Alternaria alternata* tobacco pathotype isolate 0-268 grew well

on PDA at 25°C in darkness and mycelial mats covered all the media in petri dishes in two weeks. The color of fungal colonies was usually dark brown to dark olive green brown, but quite often lighter and almost white colonies sometimes appear under the same conditions with the same medium (**Fig. 1**). In such colonies, characteristic darker concentrating markings of mycelial growth were observed. In later experiments, only dark colonies were used.

The variability is well known phenomenum for fungi of genus *Alternaria*. It is a fact since the first investigations with this fungus were done. This variability includes spore shapes and size, the optimal temperature, pH and light conditions for growth and sporulation, and pathogenicity. Diversity appears even in single spore isolates. Until now the explanation for instability of *A. alternata* is not known completely. The vegetative cells of *Alternaria* are genetically multinucleate (Hartmann, 1966). During formation of conidia one nucleus (occasionally more than one) migrates into the primordium and divides to form all the nuclei in the mature spore. Migration of nucleus through the septal pore of the hypha occurs frequently. Realizing the opportunities for genetic change through mutation, nuclear migration and anastomoses whereby any mycelium may become heterokaryotic, it follows that the conidia produced from such mycelium will be genetically different. Changing of colors of colonies in our experiments could be explained by this characteristic trait of the fungus.

Conidial production. Spores were obtained only from petri dishes, which were kept under near UV light. There was no big difference between numbers of spores produced in petri dishes that were kept at

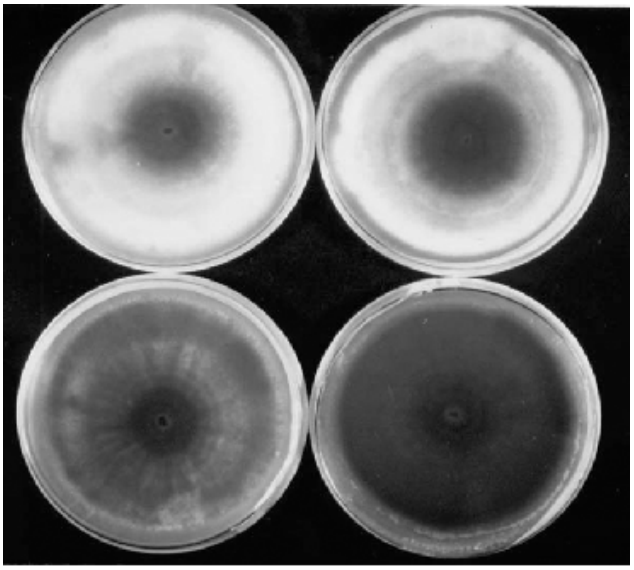

Fig. 1. Variability among colonies of *Alternaria alternata* tobacco pathotype on PDA agar medium. The color of colonies changed from dark brown (right down) to almost white (left up) under luminescent light at 25° C.

TABLE 1

Sporulation (number of spores per ml) of *Alternaria alternata* tobacco pathotype isolate 0-268 under different light conditions

Pretreatment	Light conditions			
	Continuous light	Light period (16/8 hr light/darkness)	Continuous near UV light	Continuous darkness
25° C luminescent light	0	$0.5x10^3$	$107x10^4$	0
4° C darkness	0	$0.7x10^3$	$115x10^4$	0

cool temperature in darkness and those without pre treatment in all variants of consequent light conditions (**Table 1**). No spores or only a few spores were produced on mycelial mats maintained in continuous light, light period (16/8 hrs) and continuous darkness.

As we mentioned above, the variability of *Alternaria* spp. includes also sporulation ability of the isolates. In *Alternaria alternata* tobacco pathotype as all genus *Alternaria*, the process of sporulation can be separated from that of mycelial growth (Lukens and Horsfall, 1973). Several workers have reported the optimum temperature for growth to be between 25° C and 30° C, the minimum around 5° C and maximum about 38° C (Stavely and Main, 1970; Stavely and Slana, 1971). Maximum conidial production occurs at 20° C and only a few conidia are formed bellow 16° C and above 32° C. Near UV-radiation, removal of C-source, injury to mycelium and moisture help conidiophore production. Conidial initiation is induced by cool temperatures and (or) absence of visible light. But for more isolates light is necessary for sporulation and conidia are not formed on mycelium maintained in constant darkness (Lucas, 1975). Pounds and Lucas (Lucas, 1975) found that light was necessary for sporulation and numerous spores were produced when isolates were stimulated by light/dark period, and some conidia were produced under constant light. But one isolate from Uruguay sporulated even though grown in the dark. Follstad (1966) also reported that *A. alternata* cultures grown in the dark produced spores. No spores were produced when micelial mats were exposed on continuous light, neither in contious darkness. In our case, isolate 0-268 of *A. alternata* tobacco pathotype produced large number of spores under near UV light, and few spores under light period. One period of 48 hr pretreatment of micelium by cool temperature (4° C) in darkness did not change the sporulation ability.

Inoculum potential and spore germination. Brown spot lesions developed well when concentration of 10^4 spores per milliliter was used for inoculation of detached leaves under high humidity conditions. The higher concentrations did not increase the effectiveness of spore concentration on disease development. Different workers

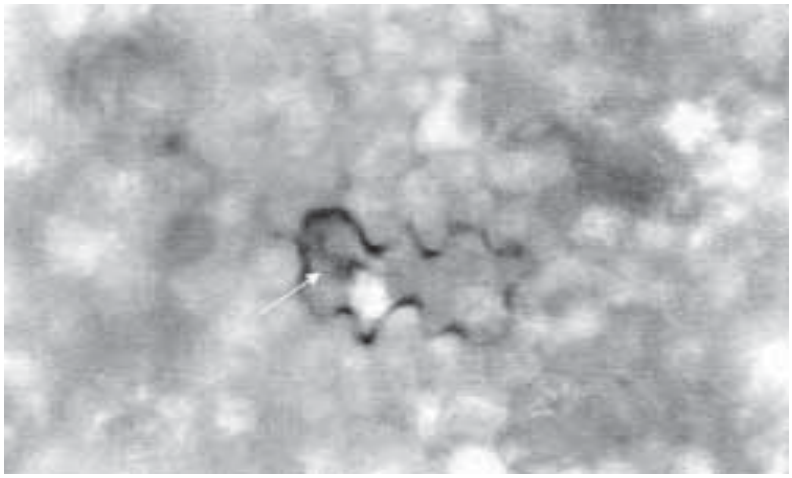

Fig. 2. The infection hypha of *A. alternata* tobacco pathotype is penetrating epidermal cell of tobacco leaf. The infected cell turns brown and dies. (Surface view x 600). Arrow indicates the spot of the penetration.

used quite large range of spore concentrations in their inoculation experiments from 5x10 to 12x10 spores per milliliter (Stavely and Main, 1970; Spurr, 1974; Stavely and Slana, 1975). Sometimes even the same researcher uses different spore concentrations in different experiments (Stavely et al., 1971). In our experiment a spore concentration of 10^4 spores per milliliter was chosen as optimal one. The difference in different works could be due to the varying virulence of isolates used and condition of inoculation tests. Laboratory studies of brown spot disease require lower concentration of spores than that in field experiments, because of better condition for infection.

The spores germinated on leaf surface in 24 hr under high humidity conditions and some very small necrotic spots could be noticed 48 hr after inoculation, when condition were favorable for infection. Observation of such points under microscope showed that germinating hyphae penetrated the epidermal cell and, as a result, the cells attacked turned brown and died (**Fig. 2**). Germinating spores of *Alternaria* produce one or more germinating tubes (Thomma, 2003). In less virulent species the germ tube targets wounds or stomata. More virulent species can penetrate directly the leaf cell (Rotem, 1994). In our experiments the germ tube of *A. alternata* tobacco patotype isolate 0-268 spores penetrates directly the epidermal plant cell, sometimes growing over stomata.

Uniformity within chosen for experiments plant pathogen isolates is essential and variability in *A. alternata* should be taken in mind before starting the experimental work. Appropriate conditions for fungal growth, conidial production and germination of spores of *Alternaria alternata* tobacco pathotype isolate 0-268 was established to precise the future experiments. Mode of penetration of the infection hypha allows investigation of the tobacco-*Alternaria* interactions.

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