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Nuclear behavior during the formation of appressoria by *Alternaria alternata*

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Nuclear behavior in the developmental process of appressoria in *Alternaria alternata* was investigated. In pregerminated conidia, approximately 94% of the conidial cells were uninucleate. The migration of a nucleus into an elongating germ tube from a germinating conidium was confirmed after 2 h of incubation at 24±1°C in PDB. Peak frequencies of binucleate and trinucleate germ tubes were detected 1 and 2 h after the peak frequency of uninucleate germ tubes, respectively. Four- and five-nucleate germ tubes did not show marked peak frequencies. A marked peak frequency of the six-nucleate germ tubes occurred about 1 h after the peak frequency of the trinucleate germ tubes, suggesting that the nuclei in the trinucleate germ tubes each divided once within 1 h. The significance of early establishment of multinucleate appressorial cells in the colonization of host plants by pathogenic *A. alternata* was discussed.

Key Words——*Alternaria alternata*; appressorium; nucleus.

Many plant pathogenic fungi produce special somatic structures known as appressoria at the tips of the germ tubes from their germinating spores. By virtue of their appressoria, they can generally penetrate artificial membranes as well as host epidermis. The appressorium is thus considered to be a fungal infection structure and has became an important target in phytopathological study (Lee and Dean, 1993). Appressorial formation is significantly influenced by some physical or chemical factors. For instance, penetration and infection by some phytopathogenic fungi producing pigmented appressoria were successfully prevented by inhibiting the appressorial melanization with nonfungicidal chemicals (Kubo et al., 1982; Yamaguchi et al., 1983; Matsuura, 1983). Thus, a thorough understanding of appressorial development and function can lead to development of strategies for controlling plant diseases.

Alternaria alternata (Fries) Keissler (formerly *Alternaria tenuis* Nees) is a ubiquitous member of the Fungi Imperfecti. It produces colorless appressoria irrespective of its virulence (Nishimura et al., 1978). Tricyclazole, an inhibitor of the biosynthesis of fungal melanin, did not inhibit the ability of the appressoria to penetrate artificial membranes or to infect host leaves (Tanabe et al., 1995). Although several studies have appeared on the nuclear behavior and cytology of this fungus (Hartmann, 1964, 1966; Nagai and Takahashi, 1966; Su and Sun, 1985), the cytological features of its appressoria were not clarified. Here, we report nuclear behavior in the developmental process of the appressoria of *A. alternata* and discuss the significance of their observed cytological features in the colonization of host plants by pathogenic *A. alternata*.

Materials and Methods

Fungal isolates Two isolates of *A. alternata* were used for the investigation: a virulent isolate (No.15A) causing black spot disease of Japanese pears, and an avirulent isolate (EGS35-193) from Simmons Culture Collection in U.S.A.

Preparation of conidia Mycelial plugs were inoculated on V-8 medium plates and incubated at 21°C under continuous near-UV irradiation (FL20S, Matsushita, Osaka) for 7 to 10 d. Conidia formed on the plates were harvested with sterile deionized water by gently shaking the plates. The conidial suspension was centrifuged at 2000 rpm for 3 min, and the collected conidia were washed once with sterile deionized water by centrifugation (2000 rpm, 3 min).

Microscopic observation of nuclei A slightly modified version of the method described by Shirane et al.(1988) was used for observing nuclei in germ tubes or hyphae from germinating conidia. Droplets of potato-dextrose broth (PDB, pH 6) containing 10^5 conidia/ml were placed on glass slides and incubated at 24±1°C for 2 to 9 h. After removal of superfluous PDB, the germlings on glass slides were fixed in Carnoy's fluid (ethanol/chloroform/acetic acid, 6:3:1, v/v) for 30 min, transferred to 95% ethanol, and kept in 70% ethanol at room temperature for 3 h. After hydrolysis in 1 N HCl for 5 min at room temperature and for 10 min at 60°C, the specimens were kept in deionized water overnight at room temperature and stained with 4% fresh Giemsa's solution (Merck, Germany) in 1/15 M phosphate buffer (pH 7) for 4 h. Subsequently, the specimens were rinsed gently with deionized water and mounted in immersion oil (Nikon).

Nuclei and appressoria were observed with a light microscope (Nikon, AFX-II) at 100× (objective lens) under oil immersion.

To observe nuclei within pregerminated conidia, conidia were bleached with sodium hypochlorite (containing 0.5% of chlorine) for 50 to 60 s and washed twice with deionized water by centrifugation (2000 rpm, 2 min). The bleached conidia were treated with Newcomer's solution (Newcomer, 1953) containing isopropyl alcohol/propionic acid/petroleum ether/acetone/dioxane (6:3:1:1:1, v/v) for 7 to 8 h. Then, the conidial suspension (5×10^5 conidia/ml) was dropped on glass slides lightly precoated with a thin layer of fresh egg albumin. After being air-dried, the glass slides with attached conidia were dipped in 1 N HCl for 5 min at room temperature and for 10 min at 60°C. Then the specimens were placed in deionized water at 4°C overnight and stained with 4% fresh Giemsa's solution for approximately 2 h prior to microscopy. The method of Shirane et al. (1988, 1989) was used to confirm the number of chromosomes observed in nuclei at metaphase.

To examine the influence of pH on nuclear number in the germ tubes or hyphae from germinating conidia of *A. alternata*, PDB media of pH from 3 to 10 were prepared. The conidia of the avirulent isolate were suspended in these media (10^5 conidia/ml) and incubated at 24±1°C for 6 h prior to nuclear staining. Nuclear number in the germ tubes and hyphae was microscopically observed as described above.

Results and Discussion

Observation of nuclei in pregerminated conidia The nuclei of *A. alternata* in pregerminated conidia bleached by sodium hypochlorite were clearly observed after HCl-Giemsa staining (Fig. 2). One conidium contained up to nine transverse septa and up to four longitudinal or oblique septa. Muriform conidia with three transverse septa and two longitudinal or oblique septa were predominantly observed for the two experimental isolates. The number of nuclei in a conidial cell was up to three (Table 1). About 94% of the conidial cells were found to be uninucleate. The shapes of nuclei within ungerminated conidia were circular (approximately 1.5 μm in diam). No significant difference in the distribution of nuclear number per conidial cell was recognized between virulent and avirulent isolates (Table 1).

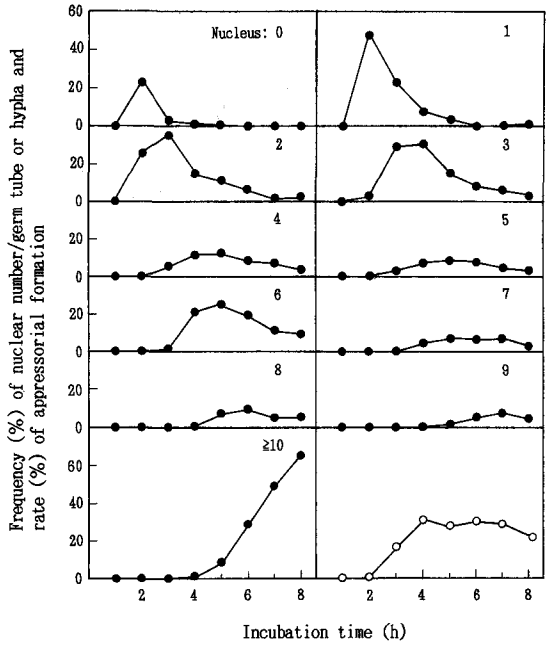


Fig. 1. Frequency of nuclear number per germ tube or hypha (●-●) and the rate of appressorial formation (○-○) in germinating conidia of *A. alternata* (No.15A).

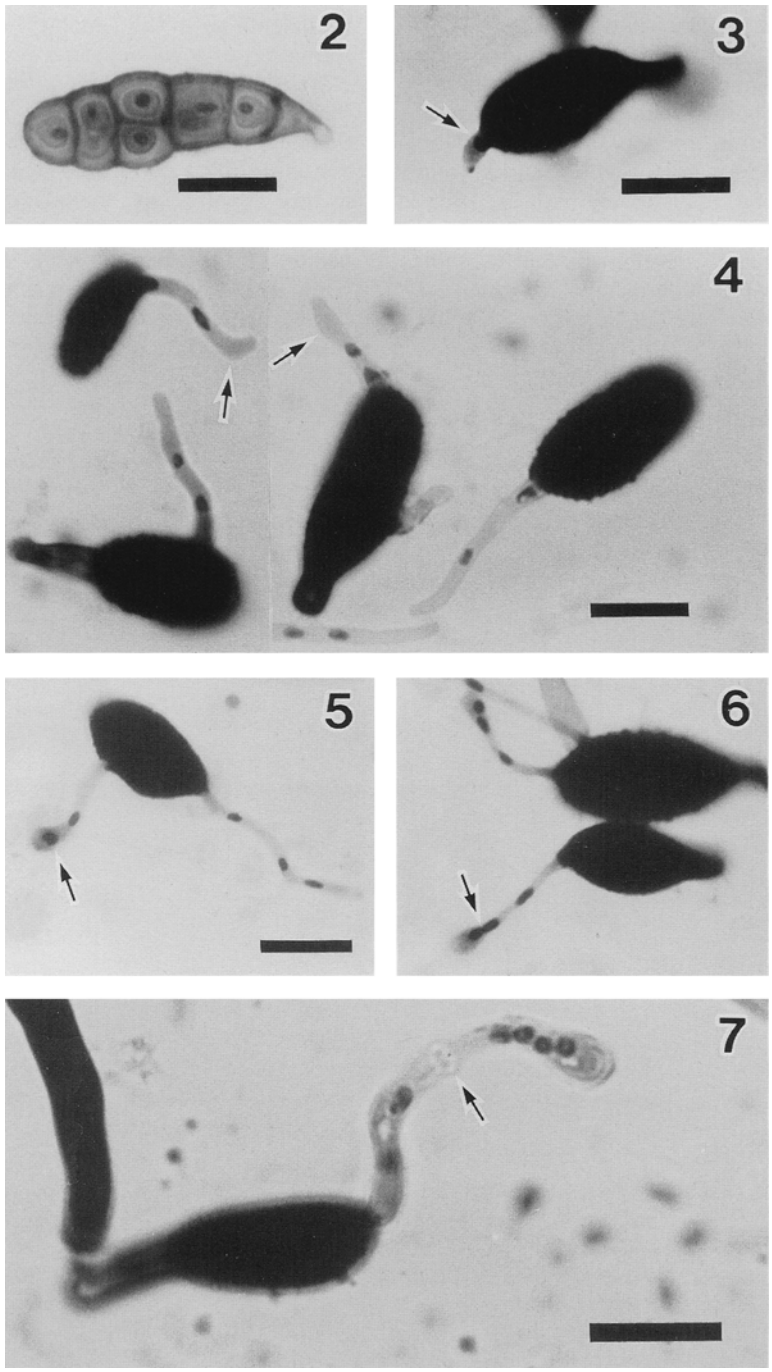
Behavior of nuclei in germ tubes and hyphae The change in frequency of number of nuclei within a germ tube or hypha developed from a germ tube of the virulent isolate is shown in Fig. 1. The peak frequency of the binucleate germ tubes was detected 1 h after the peak frequency of the uninucleate germ tubes, suggesting that the time necessary for one cycle of mitotic division of a nucleus is about 1 h under the experimental conditions. The peak frequency of the trinucleate germ tubes was detected 1 h after the peak frequency of the binucleate germ tubes, suggesting that only one of the nuclei in a binucleate germ tube divided once within 1 h. Four- and five-nucleate germ tubes did not show marked peak frequencies. A marked peak frequency of the six-nucleate germ tubes or hyphae occurred 1 h after the peak frequency of the trinucleate germ tubes, suggesting that the

Table 1. Number of nuclei per conidial or appressorial cell of *A. alternata*.

Isolate	Frequency (%)										
	No. of nuclei/conidial cell[a]				No. of nuclei/appressorial cell[b]						
	0	1	2	3	1	2	3	4	5	6	7
Virulent No. 15A	0.2	94.1	5.0	0.7	0.4	14.7	34.6	32.5	13.0	4.3	0.4
Avirulent EGS35-193	0.7	94.3	4.9	0.1	3.7	26.8	43.3	19.0	4.5	2.7	0.1

a) The number of conidial cells examined was 460 and 853 for the virulent and avirulent isolates, respectively.

b) Nuclear staining was carried out after 6 h of incubation at 24±1°C in PDB. The number of appressorial cells examined was 231 and 273 for the virulent and avirulent isolates, respectively.



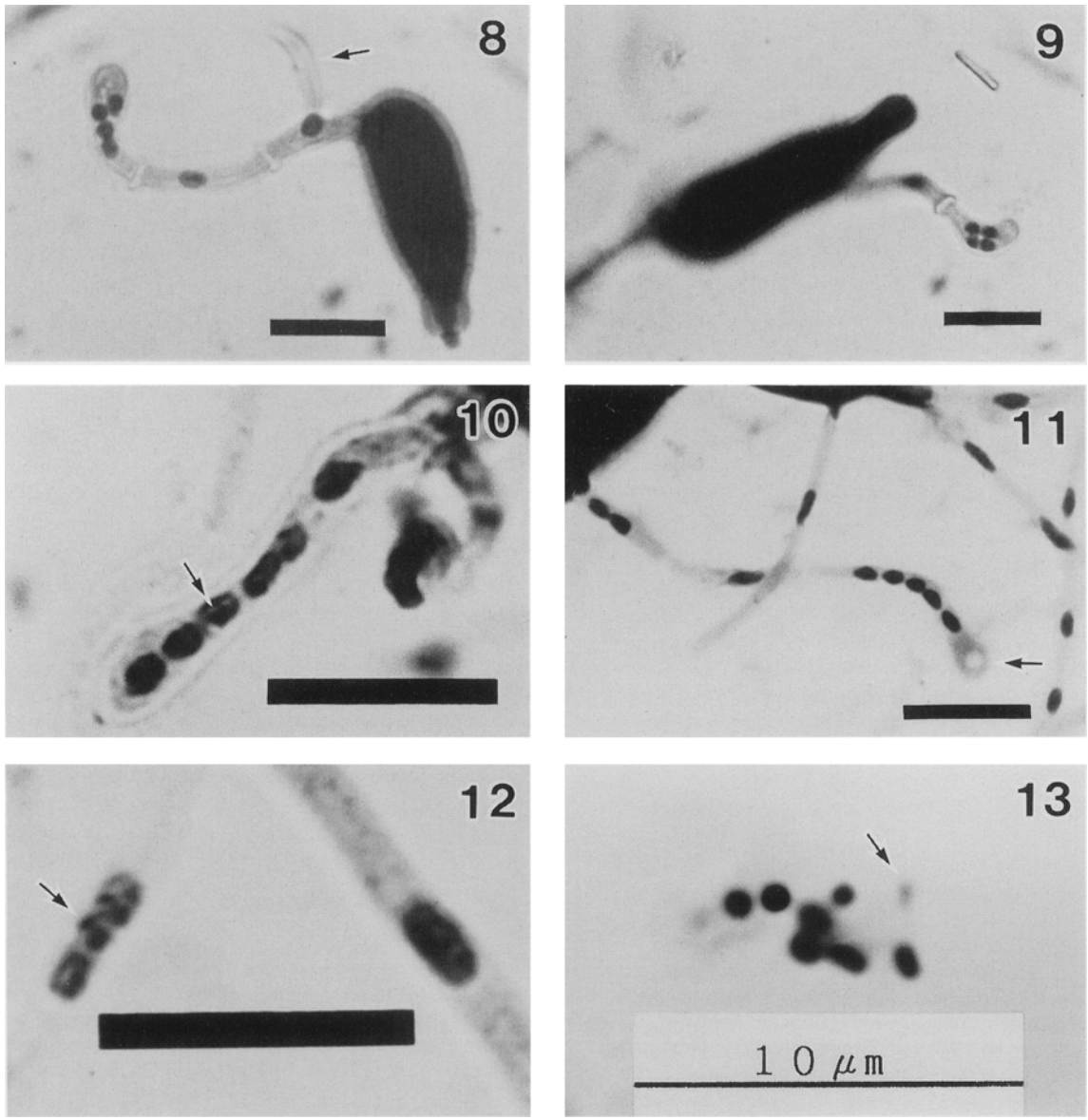
Figs. 2–7. Representative nuclear behavior in the developmental process of appressoria in the avirulent isolate (EGS35-193) of *A. alternata*. 2. The nuclei in a pregerminated conidium. 3. A nucleus (arrow) migrated into a germ tube. 4. The apexes of the binucleate germ tubes became swollen (arrows). 5. The first nucleus (arrow) migrated into the spherical appressorium. 6. The first nucleus (arrow) was dividing into 2 daughter nuclei. 7. A septum (arrow) was formed in a six-nucleate germ tube. The time of incubation was 2–5 and 6 h for Figs. 3–6 and 7, respectively. All scale bars = 10 μm. The scale of Fig. 6 is identical with that of Fig. 5.

nuclei in the trinucleate germ tubes each divide once within 1 h, resulting in the formation of the six-nucleate germ tubes or hyphae. The avirulent isolate showed the same pattern of change in frequency of nuclear number within a germ tube or a hypha developed from a germ tube as the virulent isolate did (data not shown).

Representative nuclear behavior in the developmental process of the appressoria produced by the avirulent isolate is shown in Figs. 3–7. A nucleus migrated into a germ tube from a germinating conidium after 3 h of incubation (Fig. 3). One side of the germ tube near its apex became swollen after 4 h of incubation (Fig. 4). At this

time, the germ tube frequently carried two nuclei, one located at the center and the other at the bottom of the germ tube. The swollen part of the germ tube later developed into an appressorium. After 5 h of incubation, the first and second nuclei in the appressorium-forming germ tube seemed to migrate to the appressorium and the center of the germ tube, respectively (Fig. 5). Subsequently, the first nucleus divided, resulting in the formation of a trinucleate germ tube (Fig. 6). In general, septation did not occur within a germ tube with three or fewer nuclei. After 6 h of incubation, all the nuclei in the trinucleate germ tube each divided once, resulting in the formation of a six-nucleate germ tube, and almost concurrently the initial septation occurred in the germ tube (Fig. 7).

Behavior of nuclei in appressorial cells An appressorium-forming germ tube was usually divided into two to four somatic cells including an appressorial cell after 6 h of incubation. The appressorial cell was the apical cell of the septate germ tube, which looked club-shaped (Figs. 7–11). The swollen part of this cell was the appressorium. The non-appressorial cells frequently car-



Figs. 8–13. Multinucleate appressorial cells and chromosomes of *A. alternata* (EGS35-193). 8. A mixed type of nuclear arrangement in an appressorial cell. Arrow shows a hyphal branch developed from the septate germ tube. 9. A parallel type of nuclear arrangement in an appressorial cell. 10. A linear type of nuclear arrangement in an appressorial cell. Arrow shows the intranuclear spindle. 11. A Giemsa-negative-staining structure (GS) (arrow) in a full-grown appressorial cell with linear type of nuclear arrangement. 12. Eight chromosomes in a nucleus (arrow) at metaphase. 13. Eight chromosomes from a broken nucleus. Arrow shows a faintly stained chromosome. The time of incubation was 2 h for Fig. 13; 6 h for Figs. 8, 9; 7 h for Figs. 10, 12; and 9 h for Fig. 11. All scale bars = 10 μm.

ried one to three (av. two) nuclei per cell. An appressorial cell generally contained more nuclei than a non-appressorial cell (Figs. 7, 8). The number of nuclei in an appressorial cell varied from one to seven (Table 1). More than 60% of the appressorial cells carried three to five nuclei (Table 1). The virulent isolate showed higher frequencies of multinucleate appressorial cells. However, it was difficult to conclude that the different rates resulted from specific features of the virulent and avirulent groups.

Various arrangements of nuclei within the appressorial cells were observed. These were roughly grouped into 3 types: linear (Figs. 7, 10, 11), parallel (Fig. 9) and mixed (Fig. 8). The existence of different types of nuclear arrangement indicates that there are at least two directions of somatic nuclear division in the appressorial cells. Successive nuclear divisions in the same direction might result in the linear type of nuclear arrangement, while divisions in two markedly different directions might produce the parallel or mixed types of nuclear arrangement. In the non-appressorial somatic cells of the same septate germ tubes, however, only the linear type of nuclear arrangement was observed. On the other hand, an intranuclear spindle was frequently observed in some nuclei within the appressorial cells of *A. alternata* (Fig. 10), suggesting that the mitotic division of the fungus might be a two-track type as described by Day (1972) in other fungi.

A spherical GS (Giemsa-negative-staining structure) measuring 1–2 μm in diam was frequently observed in a full-grown appressorial cell (Fig. 11). When the cell resumed growing, the GS migrated to a newly-developed hyphal apex with a low growing speed but not to one with a fast growing speed (data not shown). Since the appearence of the GS was specific for a full-grown appressorial cell, this cellular structure might be used as an indicator of a mature appressorial cell, even though its function remains to be determined. Further studies on its development and function might give valuable information for a better understanding of appressorial differentiation.

The growth of eukaryotic organisms seems to be associated with an extensive interchange of materials between nuclei and cytoplasm (Buller, 1958). The existence of mutiple nuclei in an appressorial cell means that the cell possesses a large area of nuclear surface for efficient interchange of materials between the nuclei and the cytoplasm.

The germination of a virulent conidium on its host leaf implies the start of the host-parasite interaction. The germinating conidium of pathogenic *A. alternata* is known to release inducers as well as host-specific toxins (Hayami et al., 1982). The former can induce the production of an infection-inhibiting factor in host plants, which suppresses the penetration by pathogenic *A. alternata* into the host cells (Kodama, 1989), and the latter plays a role of the initiation factor in pathogenesis (Nishimura and Kohmoto, 1983). Further, we have lately confirmed that, when one half of a young leaf of Japanese pear cv. Nijisseiki susceptible to *A. alternata* Japanese pear pathotype was pre-inoculated with the virulent conidia, the remaining half of the leaf acquired a (systemic) resistance to the subsequent infection by this pathogen 12 h after pre-inoculation at 25°C (unpublished data). Therefore, rapid infection by the pathogenic *A. alternata* seems to be advantageous to avoid the induced resistance in host plants.

It has been genetically demonstrated that a high gene dosage (copy number) of the corresponding genes leads to high yields of gene products (Gelfand et al., 1978; Rao and Rogers, 1978; Uhlin et al., 1979). Cutinase production by the virulent isolate and its involvement in pathogenicity to the host plant have been confirmed (Tanabe et al., 1988a, b). Therefore, the significance of multiple nuclei in an appressorial cell of *A. alternata* at the initial stage of conidial germination (Table 1, Figs. 7–11) might be to provide a high gene dosage (copy number) in the cell for efficient and dynamic production of the enzyme(s) necessary for fungal penetration of the host cells.

The migration of nuclei through a septal pore has been reported in the germlings of *Botrytis cinerea* Pers. ex Pers. (Shirane et al., 1988), suggesting that the number of nuclei in the appressorial cells of this fungus might be increased in two ways: by mitotic division of nuclei within the appressorial cells, and by migration of nuclei through the septal pores from other cells. In contrast, no event of nuclear migration through septal pore was observed in the germlings of *A. alternata*. Therefore, successive mitotic division of the nucleus that initially entered the appressorium appears to be the only way for the number of nuclei in the appressorial cells to increase. The nuclei in the appressorial cells of *A. alternata* could be basically considered to be genetically homogeneous with the single nuclei from the conidial cells, because 94% of the conidial cells were found to be uninucleate before germination (Table 1).

Relationship between pH and nuclear number When the conidia of the avirulent isolate were incubated at different pHs, a germ tube or hypha with more than eight nuclei was observed only in the germlings incubated in PDB of pH 4 to 8 (Table 2). The peak frequencies (approximately 20 to 26%) of trinucleate and six-nucleate germ tubes or hyphae were also observed only in the germlings incubated in PDB of pH 4 to 8. The average nuclear number per germ tube or hypha was also higher than four only in this pH range (Table 2). The germlings incubated at pH 5 possessed an average nuclear number of 4.59 per germ tube or hypha, which was higher than that of germlings incubated at pH higher or lower than 5 (Table 2). The results suggest that the pH range suitable for growth of the isolate is 4–8, and pH 5 is optimal for growth.

The determination of the suitable range and optimal pH for growth of an *A. alternata* isolate by investigating the number of nuclei in its germlings has at least two merits over measuring its colony size on agar plates: 1) the time required for testing is greatly reduced, since it is possible to see the results within 1 d; 2) wider range of pH values can be tested, since the inability of agar to solidify a strongly acidic medium ceases to be a problem.

Table 2. Influence of pH on the number of nuclei in the germ tubes and hyphae of *A. alternata*.

pH	Frequency (%) of nuclei/germ tube or hypha[a]											Average[b]
	0	1	2	3	4	5	6	7	8	9	≥10	
3	2.8	31.4	37.3	15.7	11.4	1.4	0.0	0.0	0.0	0.0	0.0	2.06
4	0.4	7.4	19.5	24.8	9.9	6.0	22.1	3.9	1.4	2.1	2.5	4.08
5	0.1	5.9	12.9	20.4	13.4	7.0	25.8	5.9	2.7	2.2	3.7	4.59
6	0.4	8.8	11.8	23.3	13.0	8.7	20.6	6.5	2.3	2.3	2.3	4.31
7	0.5	9.6	15.9	20.2	8.2	6.7	25.9	7.7	2.4	1.0	1.9	4.25
8	0.4	14.1	13.7	20.8	11.8	5.9	22.4	5.1	3.0	0.8	2.0	4.03
9	1.6	15.0	20.2	32.1	8.8	6.7	13.0	2.6	0.0	0.0	0.0	3.17
10	3.6	41.2	35.6	16.0	2.1	0.5	1.0	0.0	0.0	0.0	0.0	1.29

a) The conidia of the avirulent isolate (EGS35-193) were incubated in PDB (10^5 conidia/ml) with different pH at 24±1°C for 6 h prior to nuclear staining. The number of germ tubes or hyphae observed was 206±66 for each treatment.
b) The average of nuclear number per germ tube or hypha.

Observation of chromosomes in nuclei Eight chromosomes in a nucleus at metaphase of the avirulent isolate were observed (Fig. 12). The largest two chromosomes measured about 1.6-1.7×0.5-0.8 μm and the other smaller ones about 0.5-1.3×0.2-0.8 μm. They were arranged in two parallel rows of four chromosomes along the major axis of the hyphal cell. The size of the nucleus at metaphase was about 4.4×1.2 μm. The number of chromosomes in an intact nucleus (Fig. 12) was in complete agreement with that from a broken nucleus (Fig. 13).

Hartmann (1964) observed five chromosomes in the nucleus of an *A. tenuis* isolate, *A. tenuis* being considered a synonym of *A. alternata*. Taga and Murata (1994) presented a photomicrograph of somatic chromosomes of an isolate of *A. alternata* tomato pathotype stained by a fluorescence reagent, indicating that this isolate possesses more than eight chromosomes in a nucleus. The difference in the number of chromosomes between our isolate (EGS35-193) of *A. alternata* and the above isolates of *A. tenuis* or *A. alternata* tomato pathotype may be explained as follows: 1) the individual chromosomes stained by Hartmann (1964) may have been ambiguous, which led to a misreading of chromosomal number (no photomicrographs were shown in the corresponding paper, only hand-drawings); 2) chromosome polymorphism may exsist among isolates of *A. alternata*.

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