PENETRATION AND INFECTION OF SPINACH (*Spinacia oleracea* L.) LEAF TISSUES BY *Cladosporium variabile*

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PENETRACIÓN E INFECCIÓN DEL TEJIDO FOLIAR DE LA ESPINACA (*Spinacia oleracea* L.) POR *Cladosporium variabile*

RESUMEN

Al inocular espinaca con una suspensión de conidios de *Cladosporium variabile* en una concentración de 10^6/ml, incubar a 16-26°C y con 100% de humedad relativa, el porcentaje de conidios que germinó 12 horas después de la inoculación fue de 0.9; dicho porcentaje se incrementó progresivamente hasta 80.6, después de 96 h. El porcentaje promedio de germinación fue de 29.1 para conidios de 1 célula, 67.8 para los de dos, 2.6 para los de tres, y 0.5 para los de cuatro. La penetración, la cual se llevó a cabo a través de los estomas, no se observó sino hasta 48 h después de la inoculación. El porcentaje promedio de conidios con tubos germinativos que penetraron tejido foliar fue de 23 para conidios de 1 célula, 75.1 para los de dos, y 1.9 para los de tres. Después de la penetración, se produjeron estructuras en forma de saco, de las que se desarrollaron hifas infectivas gruesas, las que crecieron inter- e intracelularmente. Hifas semejantes a los conidióforos emergieron a través de las áreas infectadas a las 96-110 horas después de la inoculación.

PALABRAS CLAVE: *Cladosporium variabile*, *Spinacia oleracea*, mancha de la espinaca, germinación, penetración, roya cabeza de alfiler.

ABSTRACT

When spinach was inoculated with a 10^6/ml conidial suspension of *Cladosporium variabile* and incubated at 16-26°C with 100% relative humidity, the percentage of conidia germination was 0.9, 12 h after inoculation, progressively increasing to 80.6, 96 h later. The average percentage germination for 1-celled conidia was 29.1, 67.8 for 2-celled, 2.6 for 3-celled, and 0.5 for 4-celled. Penetration, which took place through stomata, was not observed until 48 h after inoculation. The mean percentage of conidia with germ tubes penetrating leaf tissue was 23 for 1-celled conidia, 75.1 for 2-celled, and 1.9 for 3-celled. After penetration, vesicle-like structures were produced from which thick infective hyphae developed. These grew inter- and intracellularly. Hyphae resembling conidiophores emerged from the infected areas 96-110 h after inoculation.

KEY WORDS: *Cladosporium variabile*, *Spinacia oleracea*, leaf spot, germination, penetration, pinhead rust.

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INTRODUCTION

Cladosporium variabile (Cooke) De Vries, the causal agent of a leaf spot disease of spinach (*Spinacia oleracea* L.) also known as "Pinhead Rust" (USDA, 1960; Westcott, 1955), has been reported to penetrate leaf tissue directly by germ tubes through cuticle in artificial inoculation studies (Mathur *et al.*, 1959). Penetration through stomata, and formation of appressoria or similar structures were not observed. It took about 22 h at 18-20°C for the fungus to penetrate, and although small initial lesions appeared after 3 days, typical symptoms were observed after 6 days. Infection took place only when the atomized plants were kept in moist chamber (Mathur *et al.*, 1959). Reed and Cooley (1911) reported that during early infection sooty conidia and conidiophores could be observed through the cracks in the cuticle. The mycelium consisted of separate, branching hyphae which grew primarily in the parietal layer of protoplasm of the spinach leaf cells. Later, conidiophores proceeded vertically from the cracks in the epidermis of young leaf lesions; in older lesions from the pulvinate masses of mycelium.

Since our preliminary observations of leaf penetration by *C. variabile* were different from those mentioned above, the objective of this study was to examine penetration and infection of spinach by an isolate collected in Skagit County, Washington.

MATERIALS AND METHODS

Isolates of *C. variabile* were obtained from naturally infected spinach plants from Skagit County, WA, collected during the summer of 1986 and kept at the WWREC phytopathology laboratory. Isolation of the fungus from leaves, single spore isolation and culture, as well as the inoculation method were carried out as described by Fuentes-Davila and Gabrielson (in press). Inoculated plants were incubated in a clean plastic chamber at 16-26°C (mean 22°C), with 100% relative humidity in the greenhouse. Several leaves at random were harvested 12, 18, 24, 36, 48, 60, 72, 84, 96, and 110-118 h after inoculation. They were cut into small pieces (0.5-1 cm), fixed in 1:17, v:v, glacial acetic acid:50% ethyl alcohol for 22-24 h, cleared and stained in 10 ml of 0.125% lactophenol-trypan blue in a water bath at 85°C for 10 min. After cooling at room temperature, specimens were rinsed with water and mounted in lactophenol on glass slides; then, the coverslips were sealed with nail polish. To determine the percentage of germination and penetration, 1800 conidia were randomly observed from the leaf samples inoculated and harvested at different time intervals.

For microtome sectioning, leaves were cut into strips 1 cm wide, fixed in formaldehyde: glacial acetic acid: 50% ethyl alcohol, 1:1:1, v:v:v (FAA) for 48 h, dehydrated through a tertiary butyl alcohol series, infiltrated and embedded in 56-57°C paraffin (Jensen, 1962). Sections 6-14 µm thick were cut with a rotary microtome, the ribbons placed on microscope slides and flooded with 0.5% formaldehyde, drained, and flooded again with Haupt's adhesive, and then dried for 12-16 h on a slide warmer at 40-45°C. The sections were rinsed in two changes of xylene, absolute alcohol, 95%, 70%, 50% ethanol, stained in safranin-fast green (Jensen, 1962), and mounted in synthetic resin. Specimens were photographed with Kodak Plus X Pan film.

RESULTS

The infection process was initiated with conidial germination, with or without considerable elongation of germ tubes, and penetration of leaf tissue. The percentage of conidia germinating and showing germ tubes penetrating leaf tissue, along with their cell number are shown in table 1. Conidial germination was 0.9% 12 h after inoculation, progressively increasing to 80.6% at 96 h. At each sampling time, conidia with germ tubes 4-5 µm long were present. In several occasions, germ tubes 230 µm long were observed. Conidia generally had 1 or 2 germ tubes regardless of the cell number. Germ tubes were either branched or not. The greatest number of conidia germinating were 2-celled with an average of 67.8%, followed by 1-celled with 29.1, 3-celled with 2.6, and 4-celled with 0.5. Rarely, 5-celled conidia were seen (Fig. 1a). Penetration of leaves was not observed until 48 h after inoculation; by this time, the percentage of conidia penetrating leaf tissue was 0.9, increasing to 12% at 96 h. The average percent of conidia with germ tubes penetrating leaf tissue was 23 for 1-celled, 75.1 for 2-celled, and 1.9 for 3-celled conidia. Penetration of leaves by germ tubes took place through stomatal apertures (Figs. 1b-f), followed by the formation of vesicle-like structures from which infectious hyphae developed (Figs. 1g, and 1h, 2a and 2b). These were thicker than germ tubes (Figs. 2c and 2d), with an average of 6 µm, and having a conspicuous thick wall. The invading hyphae ramified and grew intercellularly (Fig. 2e). The number of cells affected increased with time around the point of penetration. These areas appeared macroscopically as minute round depressions 72 h after inoculation which eventually increased in diameter, and became necrotic 24-48 h later. Although sporulation was not observed, some infectious hyphae, resembling conidiophores, started to come out from the infected areas through the stomata (Figs. 2f and 2g). In few instances, structures resembling appressoria were seen on the leaf surface (Fig. 2h).

DISCUSSION

Germination of conidia on inoculated spinach leaves followed an increasing order with 0.9% 12 h after inoculation to 80.6% at 96 h. One-celled conidia were more abundant than the 3-celled. Very low numbers of 4-celled conidia were seen, and only three 5-celled conidia were observed among all the conidia sampled. De Vries (1954), and Reed and Cooley (1911) reported that the fungus produced 1-3 celled conidia, while Mathur *et al.* (1959) observed 1-4; Jacques (1941) reported 1-6, but primarily 1-4 celled conidia. The difference in conidia cell number might not be important in trying to differentiate physiologic forms, since age and environmental conditions apparently influence the development of conidia (Reed and Cooley, 1911). Conidia generally produced 1 or 2 germ tubes from polar and intercalary cells during germination, as described by De Vries. However, Jacques reported that upon germination conidia produced 1-4 germ tubes. Two-celled conidia had the highest percentage of germination followed by 1-celled conidia. This was partly due to the greater number of 2-celled conidia present in the specimens sampled. Whether other factors might influence a greater germination in the 2-celled conidia or inhibit the other type of conidia is unknown.

Under the conditions in our study, penetration of leaf tissue was first observed 48 h after inoculation. Mathur *et al.* (1959) detected penetration by the fungus 22 h after inoculation, when

plants were maintained at 18-22°C with high humidity. This indicates that the fungus is very sensitive to temperature, since in our experiments plants were incubated at 16-26°C with 100% relative humidity. Also, it has been demonstrated that optimum temperature for infection is 15-20°C, declining sharply above 20°C (Fuentes-Davila and Gabrielson, in press). Germ tubes from 2-celled conidia penetrated leaves via stomata in greater numbers than 1-celled conidia as compared to the relative percentage of germination, suggesting that 2-celled conidia possess greater ability to penetrate leaves more rapidly.

The results of our experiments clearly indicate that penetration of spinach leaf tissue by an isolate of *Cladosporium variabile* from Washington takes place through stomatal apertures. Occasionally, structures resembling appressoria were seen on the leaf surface. However, penetration of germ tubes through cuticle was never seen in cross sections. Since Mathur *et al.*, (1959) reported that penetration through stomata and formation of appressoria were never observed, it is likely that different physiologic forms of the organism exist with the capacity to penetrate spinach leaves differently. However, the possibility that behavior of the fungus might be greatly influenced by small changes in temperature can not be ignored. Development of the fungus after penetration followed a similar pattern of other fungi (Larez *et al.*, 1986; Maclean and Tommerup, 1979) by producing vesicle from which infectious hyphae formed, and subsequently grew intercellularly.

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Table 1. Behavior of *Cladosporium variabile* on spinach leaves[x]

Hours after Inoculation	Germination (%)	Penetration (%)	CONIDIA[y] Germination(%)/Type 1-C[z]	2-C	3-C	4-C	Penetration(%)/Type 1-C	2-C	3-C	4-C
12	0.9	0.0	30.4	65.2	4.3	0.0	0.0	0.0	0.0	0.0
18	1.2	0.0	30.8	66.7	2.6	0.0	0.0	0.0	0.0	0.0
24	2.1	0.0	34.1	63.6	2.2	0.0	0.0	0.0	0.0	0.0
36	16.8	0.0	12.5	78.6	7.1	1.8	0.0	0.0	0.0	0.0
48	28.1	0.9	35.1	61.0	1.3	2.6	20.8	79.2	0.0	0.0
60	34.7	1.6	29.3	65.9	4.9	0.0	8.3	83.3	8.3	0.0
72	47.2	2.0	21.9	78.1	0.0	0.0	20.0	80.0	0.0	0.0
84	70.2	6.1	40.0	60.0	0.0	0.0	38.0	62.0	0.0	0.0
96	80.6	12.0	27.7	71.4	0.0	0.0	29.1	71.0	0.0	0.0
Average	------	------	29.1	67.8	2.6	0.5	23.0	75.1	1.9	0.0

[x]A susceptible inbred spinach line was inoculated with a 10^6 conidia/ml suspension, and incubated in a clean plastic chamber at 16-26°C with a 100% relative humidity in the greenhouse.
[y]From each sample, 1800 conidia were randomly evaluated for germination, penetration, and cell number.
[z]1-C= one celled conidia, etc.

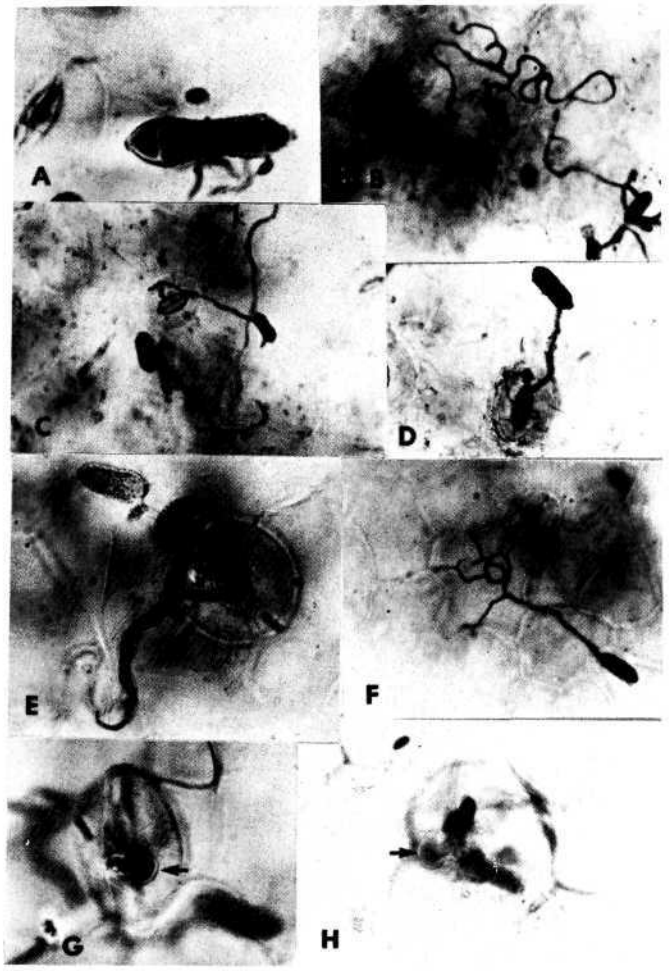

Fig. 1. *Cladosporium variabile* on spinach leaves. (a) Five-celled conidium, X 1000. (b-f) Stomatal penetration by germ tubes, b-d and f X 400; e X 1000. (g and h) Formation of vesicle-like bodies (arrows) in the substomatal chamber, X 1000. Specimens stained with lactophenol trypan blue.

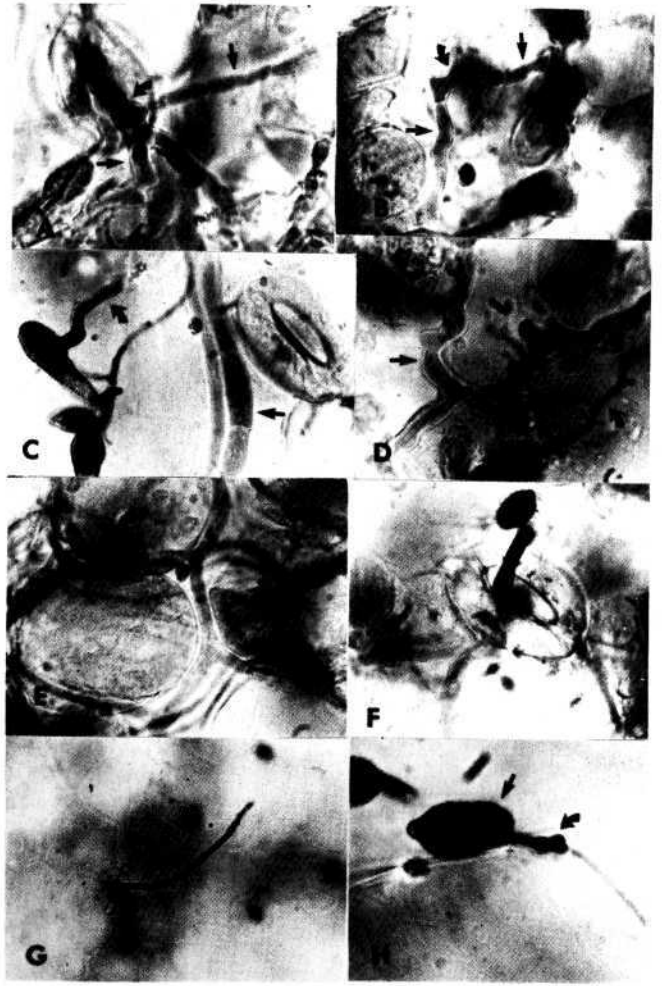

Fig. 2. *Cladosporium variabile* on spinach leaves. (a and b) Infectious hyphae (straight arrows) developing from vesicles (curved arrows), X 1000. (c and d) Infectious hyphae (straight arrows) and germ tubes (curves arrows) showing different thickness, X 1000. (e) Intercellular infectious hyphae. X 1000. (f and g) Conidiophore-like structures coming out from infected leaf mesophyll tissue through stomata, f X 1000, g X 400. (h) A conidium (straight arrow) with an appressorium-like body (curved arrow) from germ tube, X 1000. Specimens stained with lactophenol-trypan blue.