Etiology and Histology of Alternaria Rot of Persimmon Fruits

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ABSTRACT

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The causal organism of the black spot disease of persimmon fruits, *Diospyros kaki* has been identified as *Alternaria alternata*. Conidia of the fungus, pathogenic to the fruit, were found in the orchard on necrotic leaf spots. Germinating conidia were seen to penetrate the fruit cuticle directly. Following infection in the orchard, the hyphae developed intercellularly and produced tiny, dark, quiescent infections, which renewed development during postharvest fruit ripening. Latent infections increased in number throughout the growth period of the fruit. Infection via wounds also occurred in the orchard, resulting in the development of large lesions during storage. Even though germination of conidia at 0 C was only slightly delayed, the development of new infections during prolonged storage at this temperature was limited. This was probably due to the low incidence of infection by *A. alternaria* at 0 C. Black spot development was also very slow at this storage temperature. High relative humidity significantly increased the rate of symptom development and the disease incidence.

Additional key words: epidemiology, storage decay.

A black spot disease which develops in stored persimmon fruits, *Diospyros kaki* Thunb., is a serious challenge to the prolonged storage of this fruit at low temperatures. Initially, the disease was considered to be a physiological disorder of senescing fruit, which could be inhibited by high levels of CO_2 in the storage atmosphere (9). The present paper describes *Alternaria alternata* as the causal organism of this disease, showing its inoculum sources, infection periods, the histology of quiescent propagules, and storage factors affecting the propagules' further development.

MATERIALS AND METHODS

Persimmon fruits, *D. kaki* 'Fuyu,' 'Triumph,' and 'Soruga,' were used for the isolation and inoculation of the causal organism of the black spot disease. Before isolation, the fruits were disinfested with 90% ethanol. Small slices of peel with tiny or large black spots were disinfested with 0.5% NaOCl for 2 min and incubated at 25 C on potato-dextrose agar (PDA). Fungal cultures were maintained on PDA at 25 C. For fruit inoculation, single conidial cultures were used. Conidia were harvested by adding a small amount of water and gently rubbing the sporulating mycelial mat with a bent glass rod. The suspension of conidia was decanted into sterile distilled water containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20). The number of spores was determined with a haemacytometer and the concentration was adjusted to 10^6 conidia per milliliter; then 0.01 ml of the conidial suspension was placed on the fruit. Two methods of inoculation were used: without wounding, and by pricking the fruit three to five times at each inoculation site with a sterile needle after placing a drop of the suspension of conidia on the fruit. Following inoculation, the fruits were held at 25 C and covered with polyethylene bags to maintain high humidity.

Conidial germination was observed on 2% agar squares (1 cm × 1 cm × 5 mm thick) covered with a dialysis film, incubated at 100% relative humidity (RH) at 0 and 25 C. A conidium that developed a hypha twice its original length was considered to have germinated. Each of the experiments described above was repeated three times with five replicates.

Fixation and dehydration of specimens for scanning electron microscopy (SEM) and light-microscopic observations were done in a series of ethanol and acetone solutions. For SEM observations, the dehydrated tissue was handled according to the method of Cohen (6). Samples were observed in a JEOL, JSM-35C, scanning electron microscope. For light microscopic observations, the dehydrated tissue was embedded in Spurr's low-viscosity medium (19). Thin (5 μm) slices were collected in distilled water drops on a glass slide and mounted in immersion oil. Five different samples of infected tissue were observed at each stage of infection. Slices were observed with a Zeiss phase-contrast microscope.

Assessment of latent infection by *Alternaria* in the fruit during the growing season was done according to a method described by Prusky et al (16). Seven fruits were sampled from seven trees chosen at random. The fruit surface was disinfested with 90% ethanol. Disks (5 mm in diameter, 4 mm thick) were sampled from the fruit in five circular zones at different distances from the stem end: zone 1 being beneath the calyx and the following zones equidistant (0.5–1.0 cm) in the direction of the stylar end (zone 5). Twelve disks

Fig. 1. Infection symptoms of *Alternaria alternata* on fruit of persimmon cultivar Fuyu.

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were sampled from around the fruit from each zone, disinfested with 1% NaOCl for 2 min, and transferred, peel-side down, to PDA containing 40 μg/ml thiabendazole (TBZ), which is stable under sterilization conditions and selectively controls fungi other than *Alternaria*. The percentage of infected disks was determined after 4 days. The number of latent infections per fruit was evaluated according to Poisson's distribution by the tranformation $\ln(1-x)^{-1}$ (21), in which x is the proportion of infected disks obtained from one fruit.

The number of conidia transferred on persimmon fruits from the orchard to storage was determined by the dilution end-point method, after washing 10 fruits separately with 20 ml of sterile water and plating 0.5 ml on PDA amended with 40 μg/ml TBZ.

The effect of RH during storage on Alternaria rot incidence was tested as follows: fruit packed in plastic foam trays were stored at 0 C on the day after harvest in 50 × 70 × 80-cm polyvinyl chloride tents through which air streams of varying relative humidities were passed at a rate of 8.25 L/min. The relative humidities were

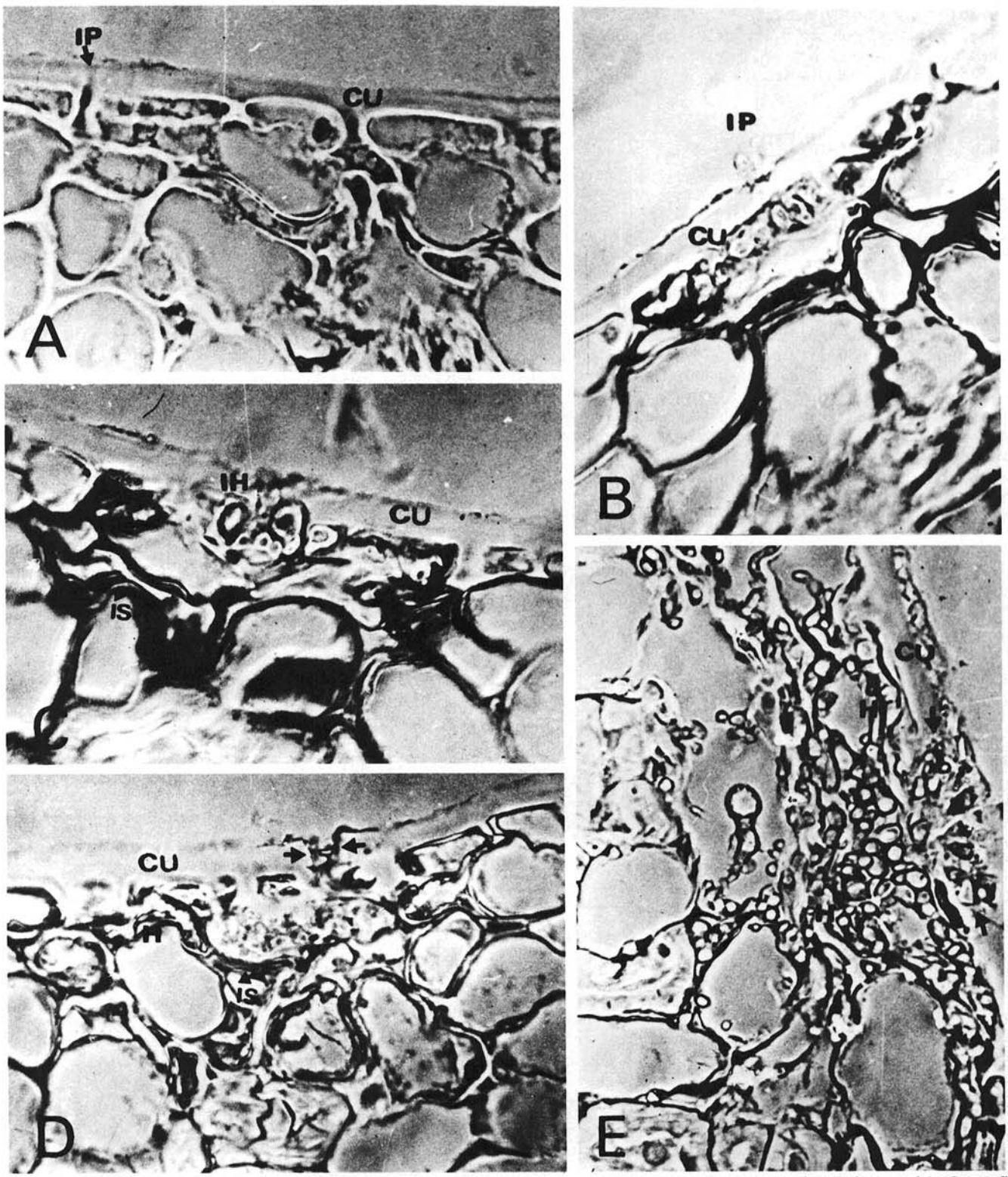

Fig. 2. Light micrographs of hyphae of *Alternaria alternata* in peel of cultivar Triumph persimmon fruits: **A–B,** infection point (IP) in the cuticle (Cu); **C,** first stages of hyphal infection (IH); note that around the penetrating hyphae, epidermal cells are destroyed and a brown color (showing up dark in black-and-white micrographs) is observed in the necrotic cells (NC) and in the intercellular space (IS) (×400); **D–E,** stages of development of the fungus; note the intercellular hyphae (H) and the sporulation through the cuticle.

obtained by drying the incoming air from the compressor over dry $CaCl_2$ to 75% RH, and passing it through six flowmeters to different saturated salt solutions as follows: $MgCl_2$ (80% RH), NaBr or $Ca(NO_3)$ (85% RH), NaCl or $Mg(NO_3)_2$ (90% RH), $NaNO_3$ (95% RH), and H_2O (100% RH). For 75% RH, the air dried over $CaCl_3$ was drawn from the flowmeter directly to the PVC tent. The airstreams entered the tents through an inlet at the bottom, passed below the fruit trays which were placed on a 5-cm-high ramp, and exited through an outlet at the top of the tent above the fruit. The RH in each tent was confirmed by measurement with a pair of dry and wet thermocouples placed above the incoming airstream. The variation in RH was ±5%. Each tent contained five replicates (cartons) of 20 fruits. The experiment was conducted twice during two consecutive years.

RESULTS

Isolation and identification of the causal organism of black spot disease. The organism isolated from infected persimmon fruits (Fig. 1) showed oboclavate spores, borne in long chains in culture, the majority with three to five cross septa and within the limits of 21–36 × 9–9.5 μm. This fungus was identified as *A. alternata* (14,18). Inoculation with conidia placed on the fruit either wounded or unwounded and held at 25 C showed characteristic symptoms of infection 3 days after inoculation. These symptoms consisted of small black spots 1.0–1.5 mm in diameter with a dark center and diffuse borders. Reisolation from the infected fruit showed *A. alternata* to be the causal organism of black spot disease in persimmons.

No pathogenic specialization was observed in isolates obtained from cultivars Fuyu, Triumph, and Soruga, and cross-inoculations developed equally well on each of the three cultivars.

Fungal penetration and symptom development. SEM observations of fruits 2–3 days after inoculation did not show any lesions in the cuticle, through which germinating conidia appeared to have penetrated. However, infection symptoms were already visible at this time. In sections observed with the light microscope, *A. alternata* was seen to penetrate directly through the persimmon cuticle following inoculation of fruit (Fig. 2A and B). During the first stages of pathogenesis, fungal development in the tissue was rather superficial (three to four cells in depth) and cellular collapse was observed (Fig. 2A and C). Darkening of intercellular spaces was always seen prior to hyphal development. Mycelium of *A. alternata* probably remains in this latent state throughout the growing season. Bursting of the fruit cuticle appeared to be the result of hyphal development and conidial production after fruit maturation (Fig. 2E). Inoculation by wounding did not induce a more rapid appearance of disease symptoms.

Sources and time of infection. *Infection in the orchard.* One source of inoculum for fruit infection in the orchard was identified on persimmon leaves (Fig. 3). Brown necrotic spots from which *A. alternata* was isolated appeared during the growing season. Inoculation of fruits with an isolate obtained from these spots caused characteristic disease symptoms. Persimmon fruits also were infected by isolates of *A. alternata* obtained from rotten apples and melons, as well as by an *Alternaria* sp. isolate from citrus and by *A. longipes*. In each of these cases, the same infection symptoms were obtained as described above.

Samples of apparently healthy disks taken from fruit peel throughout the growing season showed that latent hyphae of *A. alternata* were present at very early stages of fruit development. Small black spots 1–2 mm in diameter that were observed in the orchard during fruit growth also contained *A. alternata*. Fungal infections were concentrated mostly near the stylar end (Fig. 4). There was a nonsignificantly greater tendency for infection at the stem end. That was the reason for the sampling method adopted,

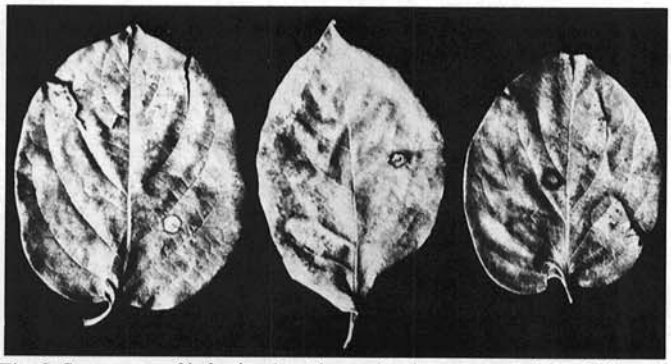

Fig. 3. Symptoms of infection by *Alternaria alternata* on cultivar Triumph persimmon leaves.

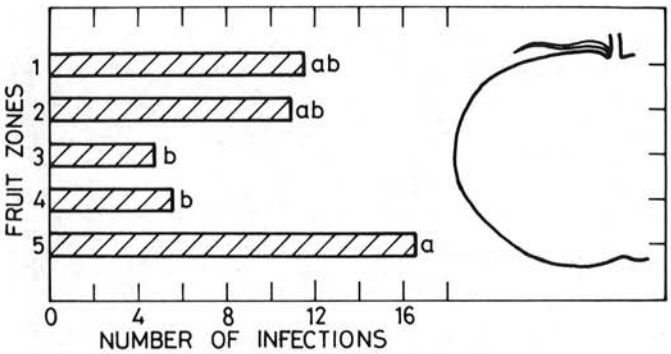

Fig. 4. Distribution of latent infection caused by *Alternaria alternata* in different zones of a cultivar Triumph persimmon fruit. Different letters indicate significantly different values by Duncan's multiple range test ($P < 0.05$).

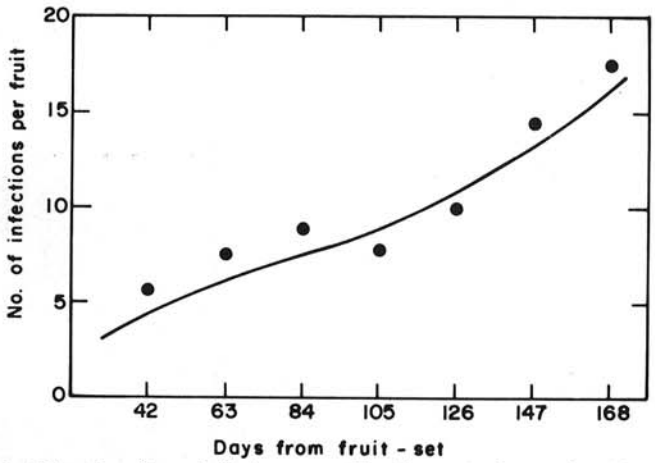

Fig. 5. Number of latent infections caused by *Alternaria alternata* in cultivar Triumph persimmon fruits during the growing season.

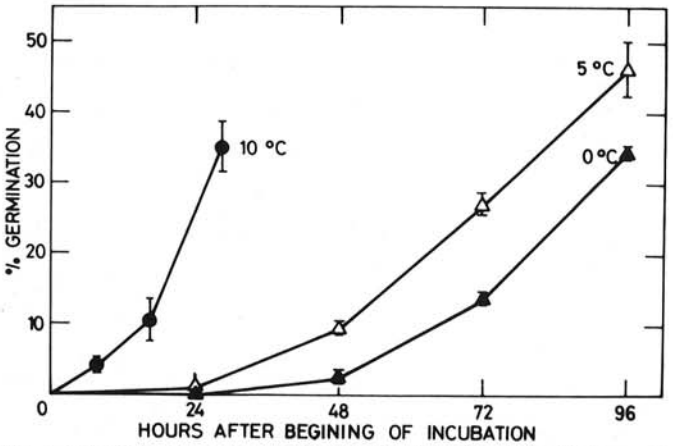

Fig. 6. Effect of temperature on the germination of conidia of *Alternaria alternata* in vitro on water agar 2% at 100% RH.

which enabled us to obtain a random homogeneous sample for each fruit representing the distribution of infection in the different zones. An evaluation of latent infections present in persimmon fruits during the 5-mo growing season is presented in Fig. 5, showing a continuous increase in Alternaria infections on fruit until harvest.

Infection during storage. Washings of freshly harvested persimmons contained an average of 100±10 conidia of *A. alternata* on each fruit. At 0 C and 100% RH, 32% of the Alternaria conidia germinated within 96 hr (Fig. 6). At higher temperatures, 5 and 10 C, the rate of germination was enhanced. The delay in germination of Alternaria spores at 0 C seems to be too small to protect the fruit from fungal attack, since persimmons are stored for up to 2 mo at 0 C.

The possibility of direct penetration by *A. alternata* during storage was investigated at two temperatures. Persimmons were inoculated with a suspension of conidia and held in saturated atmospheres at 25 and 0 C. After 3 days at 25 C, an average of 140±31 black spots per fruit had developed. On fruit held at 0 C for 10 wk, there was an average of only 0.39±0.1 black spot per inoculation point, indicating that infection by *A. alternata* during storage at 0 C is unlikely.

Effect of temperature and relative humidity in storage on black spot development. When fruit was inoculated, held at 25 C for 2 days, and transferred to 0 C, the diameter of the black spots increased very slowly during the storage period (Fig. 7). However, if the fruit was inoculated and held at 25 C in a saturated atmosphere, black spot diameters increased rapidly. Under normal storage conditions, the diameters of black spots developing from natural infections may reach 5–10 mm in diameter after 3 mo at 0 C, but after inoculation the size of the developing black spots reached only 1.5 mm. Larger black spots were obtained when persimmon fruits were inoculated and wounded (Table 1). At both 0 C and 25 C, inoculation with wounding led to a significantly more extensive development of black spots; this effect was much greater at 25 than at 0 C.

High correlation, $r = 0.85$ ($P < 0.05$), was observed between the level of cold storage RH and the extent of rot development on the fruit surface during 3 mo of storage at 0 C (Fig. 8). As expected, the loss in fruit weight during storage was inversely and very highly correlated with RH ($r = 0.997$; $P < 0.05$).

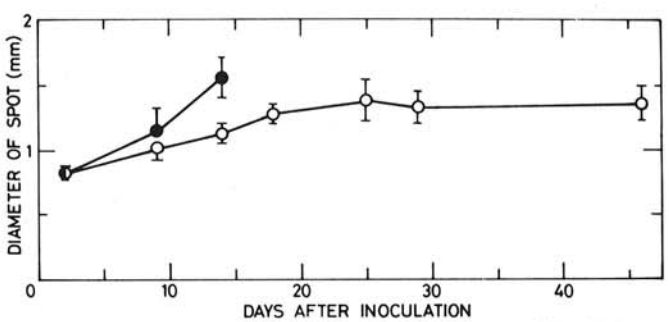

Fig. 7. Effect of storage temperature on the development of the black spot disease produced after infection by *Alternaria alternata* in cultivar Triumph persimmon fruits (0 C, o——o; 25 C, •——•). After inoculation, the fruits were incubated at 25 C for 2 days before being transferred to the indicated temperatures.

TABLE 1. Effect of different modes of inoculation on the development of black spot disease on cultivar Triumph persimmon fruits inoculated with conidia of *Alternaria alternata*

Mode of inoculation	Diameter of black spots (mm)	
	0 C[a]	25 C[b]
Without wounding	1.32 ± 0.17	11.23 ± 0.17
Wounded by pricking	8.40 ± 0.42	17.30 ± 1.18

[a] 25 days after inoculation.
[b] 3 days after inoculation.

DISCUSSION

The fungus isolated from the black spots on stored persimmons was identified as *A. alternata*. Artificial inoculation of the fruit in the laboratory produced similar disease symptoms, and reisolation from inoculated fruits proved that this fungus is the causal organism of black spot of persimmons. The incidence of *A. alternata* as a postharvest pathogen of stored fruits has increased in recent years as a result of efforts to take advantage of marketing opportunities by prolonging storage of fruits to their physiological limits.

Alternaria develops saprophytically on nonliving organic matter in the grove, on twigs, and in the soil (12). It was also found to be a common fungus on persimmon leaves and was present on the surface of harvested fruits. Conidia of *Alternaria* from brown necrotic spots of persimmon leaves could infect fruits and produce typical disease symptoms. Assessment of latent infections indicated that infection occurred during the entire period of fruit growth. Similar continuous natural infection by *A. alternata* has been found in mangos and apricots (13,16) during fruit growth. Infections by *Alternaria* were concentrated near the stem and

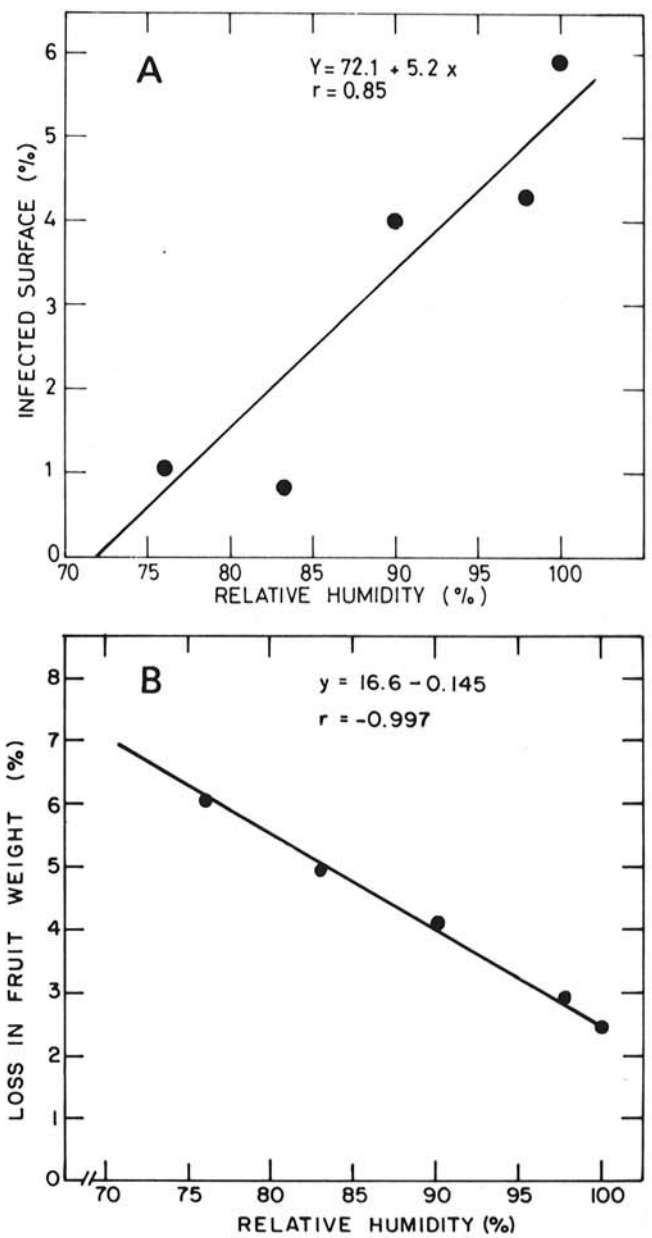

Fig. 8. Effect of relative humidity level on **A**, the percentage of fruit surface infected with black spots, and **B**, the loss in fruit weight, after 3 mo of storage at 0 C.

mainly at the stylar end. This distribution could be the result of a heavy dew deposit on the stem end of the fruit and a coalescence of droplets at a point of runoff at the stylar end of the fruit, while the middle remained practically dry.

Direct penetration seems to be a characteristic of the genus *Alternaria;* this means of infection also has been observed with *A. solani, A. cucumerina*, and *A. longipes* (11,17,22). *A. alternata* hyphae penetrate through stomata in apricots (13), but direct penetration through the fruit cuticle, as demonstrated in persimmons, has not been reported as a common mode of infection. The most common path for infection by *Alternaria* is through wounds produced before, during, or after harvest, as observed on tomatoes, blueberries, and apples (2,5,15). This type of infection also has been observed on persimmons, but it does not appear to be the principal means of ingress. Mycelium of *Alternaria* probably remains in the intercellular spaces in a latent state throughout the growing season until fruit ripening or tissue senescence, and then renews its development. Latent mycelium of *Alternaria* also has been observed in the stylar or button tissues of developing citrus fruits (1,4,12) and in lesions produced in green tomato fruits (15). Development of the pathogen in the intercellular spaces of persimmon fruits occurred following intercellular darkening and cell collapse.

Infection during storage seems to be negligible, even though conidial germination occurred at 0 C. Black spot development was relatively slow at 0 C, but higher temperatures and high RH enhanced black spot development. In vitro experiments with *A. alternata* (15) have shown a direct effect of relative humidities on conidial germination. In citrus fruit also, high RH increases the level of stem end rot caused by latent Alternaria infections (3). High RH during storage seems to affect development of rots in different ways. In some cases, it may prevent decay by enhancing healing of superficial injuries and maintaining turgidity (8,10,20). In persimmons and other fruits and vegetables (8,23), high RH seems to promote fungal development.

A possible explanation for the effect of RH in naturally infected persimmons might be that low RH in the storage atmosphere at 0 C causes loss of moisture, resulting in a higher water potential in the intercellular spaces where the fungal hyphae tend to grow (7). Fungal mycelium inside the fruit would be at a water potential in equilibrium with that of the cells (or intercellular spaces) of the fruit. Whether or not growth occurs at these sites would be determined by whether the water potential of the site is above the minimum required for mycelial growth. Fungal growth is inhibited in the range of −40 to −50 bars water potential (7). This water potential is obtained between relative humidities of 98 and 92% at 25 C (7). Former work has shown that at 96.5% RH and 5 C, germination of *Alternaria* was totally inhibited (15). Although we do not know the water potential that will prevent growth of *A. alternata* in persimmon tissue, the increase in water potential, which probably occurs within the fruit during storage at low humidity, as indicated by the loss in fruit weight, is likely to be the cause of delayed fungal development. At higher relative humidities, moisture loss was retarded and fruit turgidity was maintained, which would enable mycelial growth and development.

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