Aspergillus Colonization of Indian Red Pepper During Storage

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ABSTRACT

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The mycoflora on the surface of dried red pepper from India included several *Aspergillus* spp. When stored at 70% relative humidity (RH) in an incubator at 28 C for 10 days only members of the *A. glaucus* group developed. Infection sites, subsequent penetration, and eventual colonization of stalks, pods, and seeds were observed by scanning electron microscopy. Germ tubes from conidia gained entry to the stalks through stomata. Infection of pods occurred through crevices caused by mechanical damage. This was followed by colonization of the inner fruit wall. Healthy, intact seeds were susceptible to *A. halophilicus* which invaded via appressoria and infection pegs. When peppers were stored under high relative humidity, the predominant fungal species changed completely. *A. flavus* and *A. ochraceus* predominated at 85% RH, whereas *A. flavus* alone predominated at 95% RH. Aflatoxin production correlated with *A. flavus* growth during storage and is thus a function of relative humidity. Extent of colonization of the surface of the pepper pods was related to the relative humidity of storage; no surface growth was observed at 70% RH. The surface of the pepper pods supported visible growth of hyphae and conidiophores developed only after storage at 85 and 95% RH.

Species of *Aspergillus*, particularly those in the *Aspergillus glaucus* group as defined by Thom and Raper (21), are the most important fungi that induce storage rots in stored grains (6). This report treats the mechanism by which the *A. glaucus* group induces deterioration of Indian red peppers (*Capsicum annuum* L.) during storage at 28 C under a relative humidity (RH) of 70%.

Christensen et al (5), Pal and Kundu (12), and Flannigan and Hui (9) found *A. flavus* to be a predominant component of the mycoflora of red pepper, and Scott and Kennedy (17) found aflatoxin in red peppers of Indian origin. Because *A. flavus* requires a high relative humidity for growth (2), red peppers were stored at various relative humidities to study the growth of *A. flavus* and aflatoxin production.

Following the suggestion of Preece (13), scanning electron microscopy (SEM) was employed to determine the sequence of events during the invasion of *C. annuum* by various rot-inducing species of *Aspergillus*.

MATERIALS AND METHODS

A 5-kg package of red peppers was obtained from a warehouse in Cochin, India, and shipped by air to Canada. The initial mold contamination was determined by an analysis of samples, each containing 10 g of whole pods. Each sample was homogenized in a Waring Blendor with distilled water and appropriate dilutions were plated in potato dextrose agar (PDA) (Difco, Detroit, MI 48232) amended with 30 mg/L of tetracycline hydrochloride. The total propagule count was obtained after incubation for 3 days at 28 C. The plates were then incubated for an additional 4 days, after which the predominant colonies were subcultured on PDA and subsequently identified as *Aspergillus*, according to the group system of Raper and Fennell (15). Identification of the *A. flavus* group was confirmed with Aspergillus differential medium (1), and *A. halophilicus* was identified by the method of Christensen et al (7).

The influence of relative humidity on colonization of red peppers by indigenous *Aspergillus* spp. was studied at 70, 85, and 95% RH. To establish the desired relative humidities, each of three 10-gallon glass tanks was equipped with a Wheaton staining jar containing 100 ml of 25% NaCl; plus two or four water-soaked sponges to maintain the higher values of 85 and 95% RH which could not be attained by salt solutions alone. Pepper samples introduced to the tanks were contained in 150 × 15-mm petri plates (50 pods per plate, 10 plates per chamber). The tanks were fitted with Plexiglas

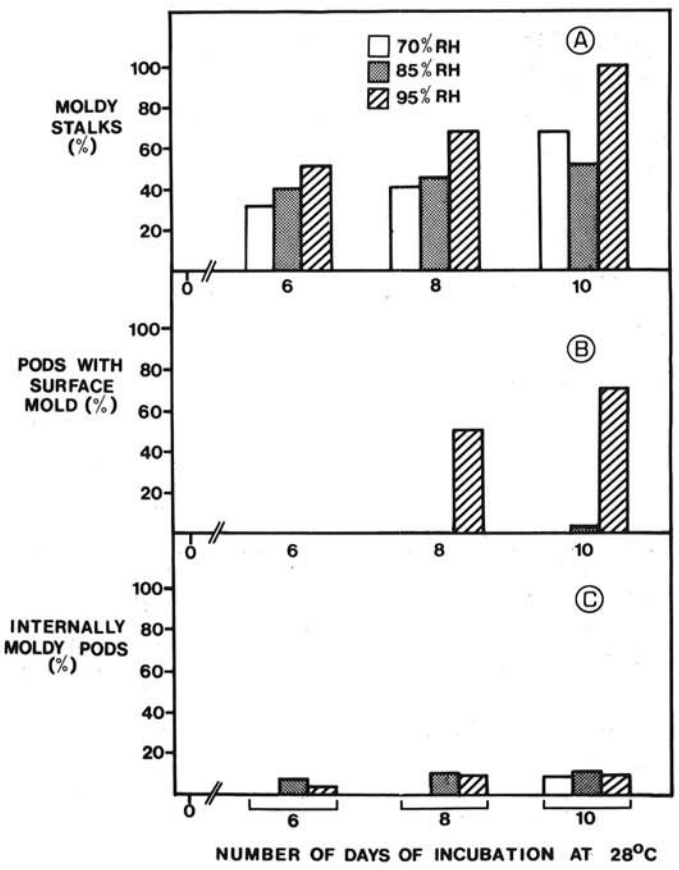

Fig. 1. Effect of relative humidity levels on sites of colonization of dried red peppers by *Asperigillus* spp. during storage.

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lids that were edged with weatherstripping to provide a tight seal and placed in incubators at 28 C. The RH in each tank was recorded continuously with a hygrothermograph (Model 22-7078; Bacharach Instrument Co., Pittsburgh, PA 15238). The desired relative humidities remained constant during the 10-day incubation period.

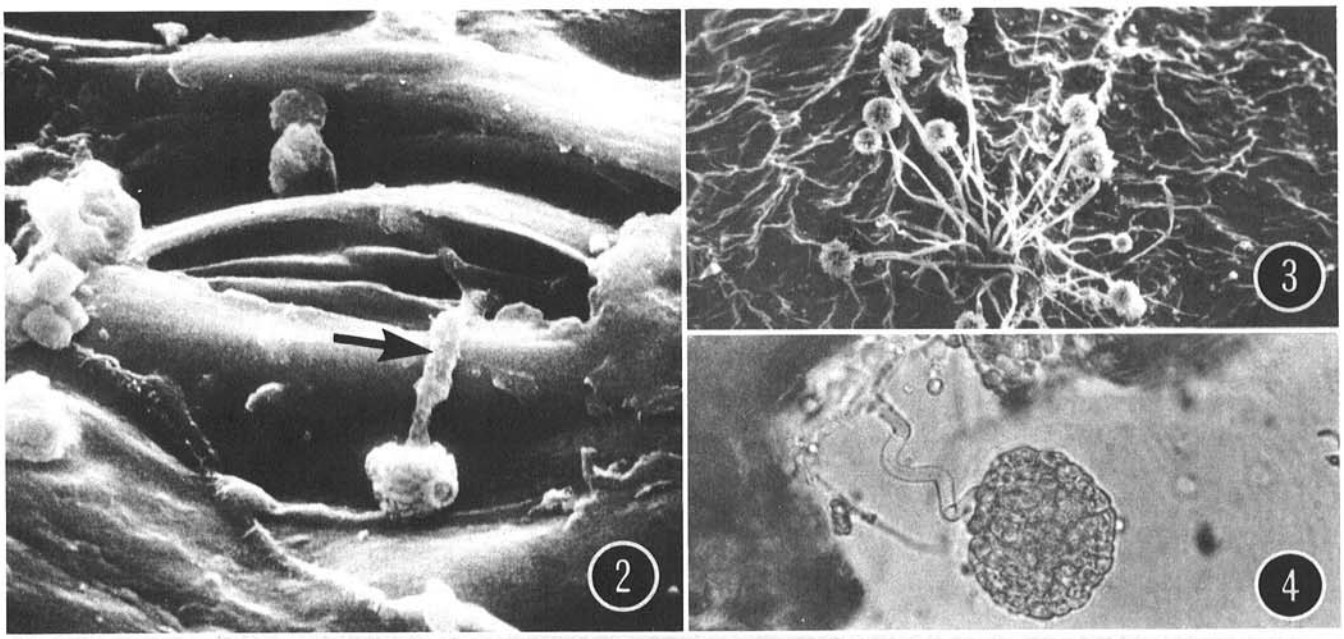
Extraction of aflatoxin was carried out according to the procedure of Holaday and Lansden (10). However, the addition of 5 g of Celite (Johns-Manville Products Corp., Denver, CO 80110) to each 25 g of sample prior to blending in 50 ml of methanol: water (4:1, v/v) mixture greatly improved sample clarification. The minicolumn method for aflatoxin detection (11) was significantly modified for spices in order to eliminate interference from pigments and other natural substances (18). For samples giving a positive result on the modified minicolumn, 20 μl of the benzene extract was spotted on 20 × 20 cm silica gel G chromatography plates (Supelco, Inc., Bellefonte, PA 16823), along with 5, 10, and 15 μl of aflatoxin standards (Supelco, Inc.) containing 1 μg/ml of B_1 and G_1, and 0.3 μg/ml of B_2 and G_2. The plates were developed according to the procedure of Suzuki et al (20) and observed under long-wave (no shorter than 300 nm) ultraviolet light. The results given are estimates based on visual comparison to internal standards, derived from the analysis of triplicate samples.

To examine conidial germination, penetration, and colonization of red pepper, segments of pods were placed directly on aluminum stubs and coated with a 30-nm gold film in an International Scientific Instruments (ISI) PS-2 sputter coater (International Scientific Instruments, Inc., Mountain View, CA 94043). Specimens were observed in an ISI Super 3A scanning electron microscope operated at 25 kV.

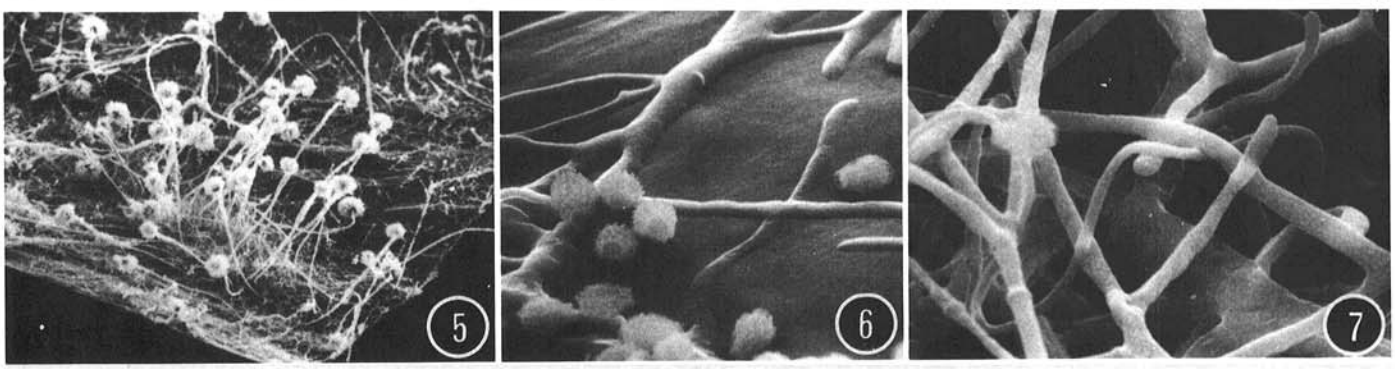
RESULTS AND DISCUSSION

Mycoflora of sample before storage. Prior to warehouse storage, *Aspergillus* spp. isolated from dried peppers having a total mold count of 6.0×10^5/g included, in order of frequency, members of the *A. flavus, A. niger, A. glaucus,* and *A. ochraceus* groups. A representative isolate of the *A. glaucus* group was identified as *A. halophilicus* by its osmophilic nature, form of conidia, and cleistothecia as previously described (7).

Mechanism of *A. glaucus* group infection. There was no visible mold growth on any part of the dried pods at the beginning of storage (Fig. 1). As anticipated from reports of fungi detected in stored grains (6), only the *A. glaucus* group was found during storage at 70% RH. The discussion of the mechanism of infection by this group therefore involves the stalks on which mold growth



Figs. 2–4. Micrographs of stomatal entry and stalk colonization in red pepper by *Aspergillus halophilicus.* **2,** Scanning electron microscopy (SEM) of conidial germination and site of germ tube (arrow) entry at the stoma on red pepper stalk (× 3,450). **3,** SEM of a cluster of conidiophores arising at the stomatal region (× 81). **4,** Photomicrograph of a cleistothecium arising from a red pepper stalk (× 575).

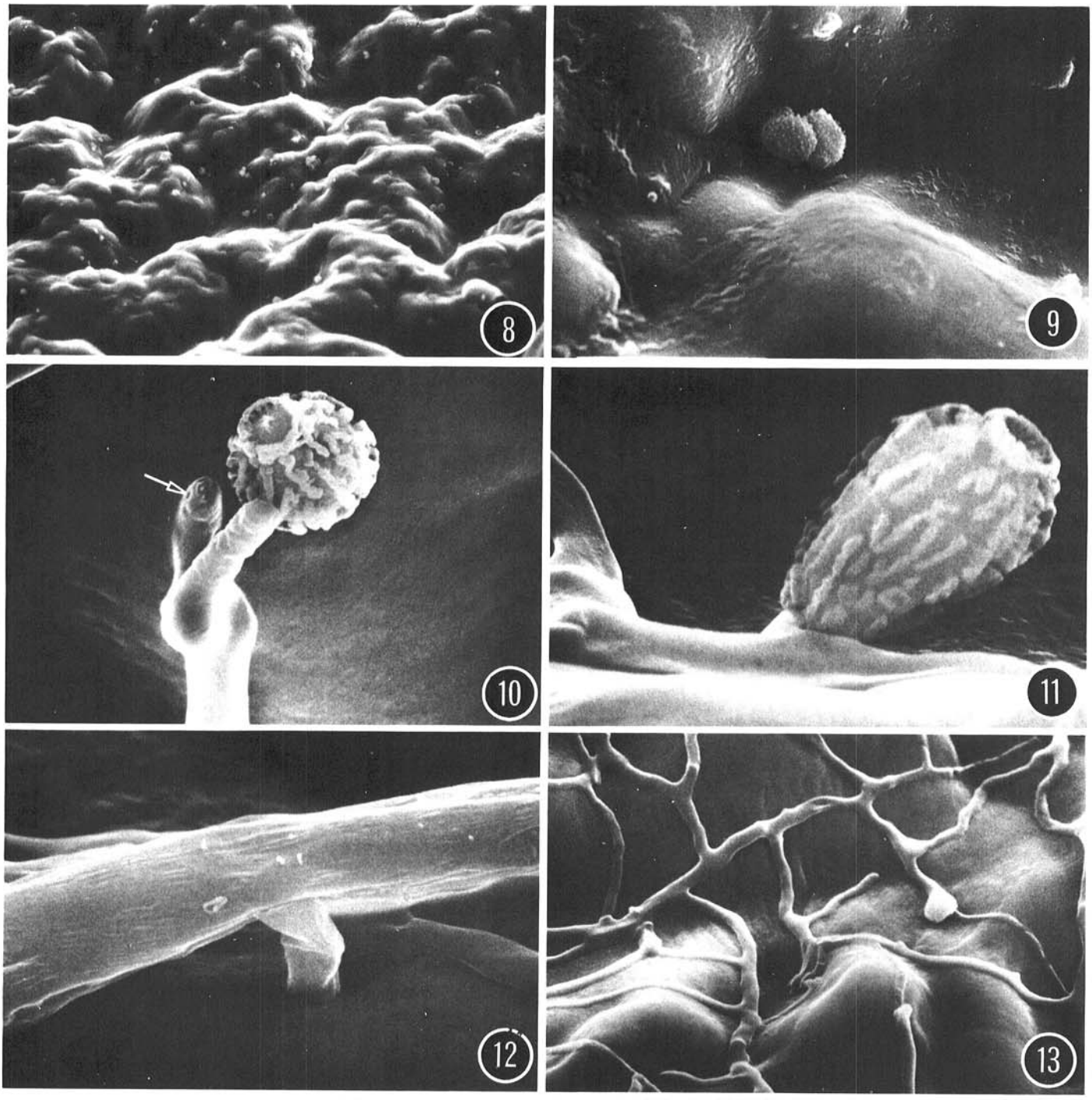


Figs. 5–7. Scanning electron micrographs of the inner fruit wall of red pepper showing events during colonization by *Aspergillus halophilicus.* **5,** Well-formed conidiophores (× 30). **6,** Conidia and mycelium showing regions of hyphal contact with the fruit wall (× 2,000). **7,** Established mycelial network (× 2,200).

increased during storage under all three levels of relative humidity (Fig. 1A). It does not involve growth on the surface of the pods, which only occurred at 85 and 95% RH (Fig. 1B). Internal mold growth was limited to a small percentage of pods that had sustained mechanical damage at some stage prior to storage; most likely during drying or packaging. Internal mold occurred at all three levels of relative humidity (Fig. 1C), including *A. glaucus* growth after 10 days of storage at 70% RH.

Successful colonization of the red pepper stalk depended upon the presence of numerous stomata, which were readily accessible penetration sites for the germ tubes arising from conidia (Fig. 2). Although conidiophores were produced over the entire surface of the stalk (Fig. 14), the stomatal region was the primary site for the production of clusters of conidiophores (Fig. 3). Members of the *A. glaucus* group produce cleistothecia in laboratory culture media (15). Observation of sections of red pepper stalks under the light microscope revealed well-formed spherical cleistothecia attached to distinct spiral stalks (Fig. 4).

Numerous conidia were produced following the colonization of the stalk. Hyphae from the stalk portion extended across the surface of the pod, with sparse production of conidiophores. These hyphae intruded through the crevices of pods that had suffered mechanical damage and produced a dense mat of conidiophores on the inner fruit wall surface (Fig. 5). This growth could only be



Figs. 8–13. Scanning electron micrographs of red pepper seed showing fungal colonization. **8,** Seed surface with dispersed conidia (× 58). **9,** Conidia deposited in minute depressions on seed (× 575). **10,** Appressorium with pressure points (arrow) and a well-formed primary hypha from a conidium of *Aspergillus halophilicus* (× 7,935). **11,** Conidium of *A. halophilicus* with a basal polar germ tube (× 10,350). **12,** A penetration peg arising from a primary hypha of *A. halophilicus* on the seed coat (× 11,500). **13,** An array of the primary mycelium of *A. halophilicus* on the seed surface (× 2,300).

attributed to hyphae having first established intimate contact with the surface of the inner fruit wall (Fig. 6). No germination of newly produced conidia was observed on the inner fruit wall. Actively growing mycelium inhibits conidial germination of members of the same species (3) and may represent an example of innate inhibition (4,16). This mechanism could allow an ecological conservation of conidia, enabling conidia to occupy noncolonized pods when dispersal is achieved. Dispersal of mold in red pepper by insects, such as beetles and moths, has been demonstrated (19). Hyphae from germinating conidia had extensive lateral branching with numerous anastomoses; the profuse aerial branching formed a massive three-dimensional network of mycelium (Fig. 7).

The outer integument of the red pepper seed is irregularly undulate with numerous convolutions and is covered by a distinct cuticle. Mechanical damage of a pod often results in the liberation of seeds, and it is common to find loose seeds in samples of red pepper. Such seeds were visibly free of mold growth. Owing to the opacity of the seed, it was difficult to see fungal conidia under the light microscope; but SEM enabled observation of conidia on the seed surface (Fig. 8). Conidia, especially the echinulated ones, were lodged and retained in narrow depressions on the seed surface (Fig. 9).

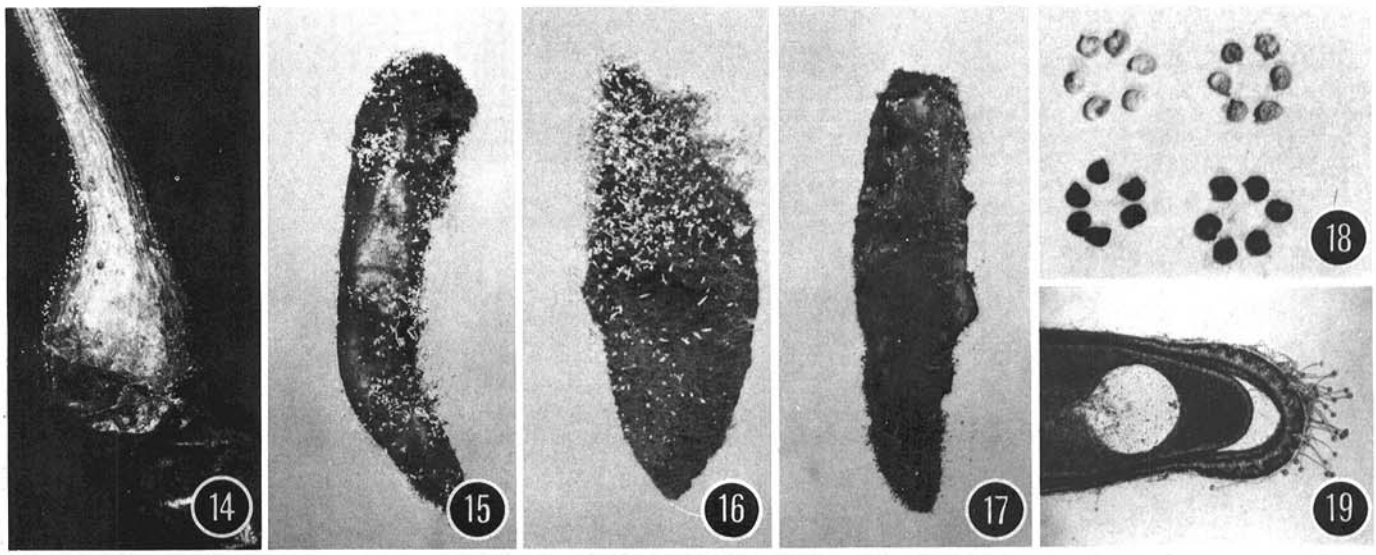
Colonization of seeds contained in mechanically damaged pods appeared to follow production of conidia within the pod. Plating these seeds on any laboratory medium, although proving contamination, would not allow light-microscopic observation of conidial germination and colonization on the seed. SEM was useful for observing conidial germination, penetration, and colonization in situ. Germ tubes arising from conidia, following a short growth period, terminated in ovoid appressoria (Fig. 10). Pressure points were noticeable as radial depressions on the appressorium and most likely are involved in anchoring the germ tube firmly to the integument of the seed. A central papillate tip on the upper surface of the appressorium corresponded to the position of a fine penetration peg arising from the adnate surface of the appressorium. Neither the penetration peg nor its entrance into the sclereid layer of the seed could be demonstrated by SEM; however, these were observed by light microscopy when thin sections of seeds were prepared (photograph not included). Primary hyphae were produced by the appressoria at a point perpendicular to the place of origin of the penetration peg on the seed surface. The appressorium at this stage consisted of a multicellular appressorial pad. The germ tube in most cases appeared to exit through the side of the conidium, but conidia-producing polar germ tubes also were observed (Fig. 11). Therefore, a definite, predetermined point of germ tube emergence is evidently lacking in the *A. glaucus* group. Such conidia are autotrophic (16): the emergence of the germ tube is determined by local gradients of stimulants or inhibitors and these determine where the conidial wall softens.

Unlike the growth on the surface of the inner fruit wall, the primary hyphae on the seed integument produced distinct penetration hyphae (Fig. 12). A similar hyphal penetration was reported for ramifying hyphae of *Erysiphe* (8). However, it is uncertain whether the penetration process is mechanical, enzymatic, or both. Mechanical penetration seems unlikely, because a thick layer of sclerides covers the seed integument. If mechanical penetration should occur, it would exert considerable pressure on the integument, resulting in a depression or tearing in the immediate vicinity of penetration. No such damage was apparent when seeds were examined under the SEM (Fig. 13). More likely, penetration is associated with enzymes produced at the tip of the infection hyphae (11) and, in common with other plant pathogens, that *Aspergillus* produces cellulolytic enzymes (14).

Mold growth at high relative humidity. The reason for conducting storage tests at 85 and 95% RH was to determine the influence of relative humidity on aflatoxin production by *A. flavus*. These relative humidity values are encountered frequently in Indian warehouses; and it is relative humidity, rather than a more precise measurement such as water activity, that has been used to describe conditions influencing growth of *A. flavus*. Two major changes were observed when red peppers were stored at high relative humidity. First, there was a complete change in the species of molds which predominated; and second, growth occurred on the surface of the pods (Fig. 1).

At 70% RH, growth of *A. halophilicus* was confined to the stalk (Fig. 14). At 85% RH, the same growth pattern on the stalks was observed, but the molds were predominantly members of the *A. niger* group, with fewer belonging to the *A. flavus* and *A. ochraceus* groups. At 95% RH, members of the *A. flavus* and *A. ochraceus* groups predominated; there were relatively few of the *A. niger* group. Relative humidity did not significantly influence the incidence of moldy stalks during storage (Fig. 1).

Mold growth on the surface of the pods occurred only during storage at 95% RH (Fig. 1). Figure 2 shows a pod that was covered mostly by conidial heads of the *A. ochraceus* group. In other cases growth on the surface of the pod was comprised of members of both the *A. ochraceus* and *A. flavus* groups (Figs. 15,16), or by the *A. flavus* group alone (Fig. 17).



Figs. 14–19. *Aspergillus* spp. growth on stored red pepper (× 1.5 unless indicated). **14,** *A. halophilicus* on red pepper stalk after storage at 70% RH. **15,16,** Red pepper pods after storage at 95% RH overgrown by *A. ochraceus* (white conidial heads) and *A. flavus* (dark conidial heads). **17,** Pod with predominant growth of *A. flavus* after storage at 95% RH. **18,** Seeds of red pepper: top left, healthy seeds; top right, seeds with *A. ochraceus*; bottom left, seeds with *A. niger*; and bottom right, seeds with *A. flavus*. **19,** Section of red pepper seed showing established growth of *A. ochraceus* (×80).

As previously stated, internal colonization was restricted to mechanically damaged pods regardless of relative humidity. At elevated relative humidity, the organism involved could be determined by the color of the infected seeds (Fig. 18). In normal pods the seeds were ivory white. The seeds became yellow in pods containing the *A. ochraceus* group, black in pods containing *A. niger*, and olive green in pods with *A. flavus*. Although the precise mechanism of seed infection was not determined by SEM, the changes in the color of the seed were not merely due to a dusting with conidia, but were the result of colonization followed by profuse conidiophore production as shown in Fig. 19 (*A. ochraceus*).

The crucial factor is the relationship between humidity and aflatoxin production. Although *A. flavus* was the predominant organism in the dried sample before storage, no aflatoxin had been produced. Furthermore, none was produced during storage at 28 C for 10 days at 70% RH, because *A. flavus* did not grow. Analysis of the red peppers after storage at 85 and 95% RH showed that aflatoxin B_1 had been produced to estimated levels of 90 μg/kg and 180 μg/kg, respectively. This correlates with the observation that *A. flavus* was a minor component of the mycoflora growing on red pepper at 85% RH but became a predominant organism during storage at 95% RH. Five internally colonized pods from the sample stored at 95% RH containing *A. flavus* alone and selected on the basis of internal colonization, contained aflatoxin B_1 at an estimated level of 70,000 μg/kg. The occurrence of aflatoxin at such concentrations is not uncommon (22).

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