Research Note

Infection and Ultrastructure of Conidia and Pycnidia of Stenocarpella maydis in Maize

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ABSTRACT

Stenocarpella maydis is the most prevalent ear rot pathogen of maize (Zea mays) in South Africa, the United States, and other countries. Infection and ultrastructure of propagules of S. maydis in maize were observed by light, scanning, and transmission electron microscopy. Two-celled conidia of S. maydis were found in the tissues of husk and kernels. Mycelia colonized inter- and intracellularly in the host tissues. Pycnidia were found abundantly inside the seed tissues of susceptible cultivars; within a single seed, pycnidia propagated preferentially in embryonic tissues. A pycnidium is composed of morphologically different resting spores mingled with some degraded organelles of the host cell. In this study, various enzymatic activities led to cell wall degradation, lacunae in endosperm tissues, and disrupted organelles in susceptible cultivars. In contrast, callose deposition surrounding fungal hyphae was clearly visible in resistant cultivars. Heavy infection was detected by maceration, even though there was no apparent symptom on the seed coat. The saprophytic nature and structurally different forms of propagules could contribute to a long-term survival of this pathogen in the field and during grain storage. Furthermore, S. maydis might pose a threat of diplodiotoxin intoxication to human and domestic animals when infected maize seeds are consumed.

Ear rot comprises major fungal diseases affecting the successful production of maize, since it directly influences yield, grade, and price of the final product (1, 12, 20). Stenocarpella maydis (Berk.) Sutton, formerly Diplodia maydis, is the most prevalent ear rot pathogen of maize (Zea mays L.) in South Africa, the United States, and some other countries (1, 3, 15). Furthermore, the fungus is reported to produce diplodiotoxin, which causes diplodiosis in cattle and other animals (25, 33). Another morphologically related species, Stenocarpella macrospora, with a markedly bigger, two-celled conidial spore, is more prevalent in humid subtropical and tropical zones; it produces symptoms of dry ear rot, stalk rot, and even leaf striping (23, 35).

Koehler (22) described the ramification of S. maydis infection in maize. The modes of colonization and infection of S. maydis was also described (3, 4, 8). Bensch and van Staden (4) observed that appressoria formed at the hyphal tips 72 h after inoculation, and penetration by a hypha resulted in the inter- and intracellular colonization in maize. Specifically, preferential colonization of the embryo in the kernel by S. maydis was reported (3). Molecular detection methods of the pathogen in seeds or in the field were developed (2, 11, 37). Chitinase might be involved in the interaction between host and pathogen (24). More recently, Naumann and Wicklow (32) demonstrated that susceptibility to modification of a fungal-targeted plant chitinase differs among inbred lines, suggesting that the LH82 line allele is a specific genetic determinant, contributing to resistance to the ear rot caused by S. maydis, whereas the B73 line allele might contribute to susceptibility. Many efforts via biological control (7), fungicides (10), and plant resistance (13, 32) have been made in an attempt to control the infection. The objective of this study was to observe the in situ ultrastructure of propagules of S. maydis in maize tissues to characterize the infective mechanisms of this pathogen.

MATERIALS AND METHODS

Plant materials and inoculation. Two resistant maize cultivars, RS5206 and PAN6264, and two susceptible ones, SNK2266 and PAN6140, were used throughout this study. Seeds were sown in plastic pots filled with compost. Potted seeds were maintained in an environmentally controlled greenhouse at 30°C, with 100% humidity. Each pot contained two plants. In addition, naturally infected maize seeds of cultivar SNK2266 were collected in fields in KwaZulu-Natal, South Africa.

U2 strains of S. maydis, isolated previously (37), were incubated separately in potato dextrose broth at 25°C on a shaker. The conidial suspension was adjusted to ~10⁷ spores per ml. Cultivars were inoculated 15 days after sowing. One milliliter of spore suspension of isolates was injected into the tip of the ears with a syringe. Each treatment contained six plants; experiments were performed three times. Two weeks after inoculation, healthy and diseased tissues were sampled every week.

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Samples were fixed in 3.0% glutaraldehyde for 8 h or overnight, washed twice with 0.05 M sodium cacodylate buffer, and dehydrated with an ethanol serial dilution (50%, 70%, 80%, and 95% ethanol for 15 min each time, and then by 100% ethanol [three times] for 10 min each time). Ethanol absolute (dried by copper sulfate) was used for the last change (19). Samples were critical point dried with a CPD 750 (Bio-Rad, Hercules, CA), mounted onto brass stubs, and gold coated with a Polaron SC500 sputter coater (VG Microtech, Sussex, UK). Hard kernels were mounted onto brass stubs and gold coated without any pretreatment. Samples were viewed with a scanning electron microscope (model 500, Philips, Amsterdam, The Netherlands) at 10 kV (19, 31).

Transmission electron microscopy. Husks and kernels were cut into sections 2 to 3 by 2 to 3 mm², and then fixed in 3% glutaraldehyde overnight. After two washings, samples were postfixed in aqueous osmium tetroxide, washed in distilled water, and dehydrated with a graded ethanol series (50% once, 70% once, 95% twice, and 100% twice for 30 min each time). Samples were pretreated with propylene oxide for 10 min and then subsequently infiltrated with serial resin (Spurr resin–propylene at 1:1 ratio for 12 h; 3:1 for 12 h; and finally, fresh, pure Spurr resin, overnight). Samples were embedded in silicone molds with fresh resin for 48 h at 60 to 70 °C (19, 31).

For ultramicrotomy, thin sections (80 nm) were cut with glass knives. Ultrathin sections with metallic colors (gold to silver) were picked up on copper grids, stained in uranyl acetate for 20 min, and washed twice in a distilled water jet stream. Thereafter, grids were dried and submerged in lead citrate for 10 min. Grids were then washed in a sodium hydroxide solution, then in distilled water, and then viewed with a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) at 60 kV (19, 31).

Maceration. All seeds were examined visually for infection on the seed coat before maceration. Ten seeds from a cultivar were placed in a 250-ml flask containing 50 ml of maceration solution (10% [wt/vol] sodium hydroxide and 0.5 g/liter tryptophan blue [cotton blue]) for 48 h at 25°C. Over 200 seeds were macerated for each cultivar. After alkaline treatment, the seeds were agitated gently in a water bath (65°C) for 5 min. Seeds were transferred to a beaker containing 10 ml of lactophenol. Beakers were placed in a water bath (65°C) or heated gently over a low flame until embryos were cleared (27, 34). This enabled complete detachment of the embryo from the seed coat. Presence and morphology of S. maydis in seed coats and embryos were examined with light microscopy.

RESULTS AND DISCUSSION

Severely infected maize seeds, albeit no apparent symptoms on the seed coat. Although molecular detection of S. maydis has been developed (2, 11, 37), seed maceration (27, 34) still is a quick and reliable approach to observe the natural appearance of pathogens in seeds. After maceration, embryos can be detached easily from seed coats. The infected embryos appeared brown to dark brown because of the abundant presence of mycelia and pycnidia (Fig. 1A), in contrast with the clear, transparent, healthy embryos (Fig. 1B). Some resting structures with thick cell walls were also observed among normal mycelia (Fig. 1C). For three batches (more than 200 seeds for each batch) of naturally infected seeds of susceptible cultivar SNK2266, the average infection rate by visual examination of symptoms was 39.6 ± 3.3 (standard error), in comparison with an infection rate of 45.1 ± 4.3 (standard error) revealed by light microscopy after maceration. There was a significant correlation (r = 0.84, P < 0.01) between infection rates by two parallel examination methods. However, the discrepancy resulted from severe infections (detected with light microscopy after maceration for some seeds), without any apparent symptoms on the seed coat.

Morphological observation of propagules of S. maydis. In resistant cultivars RS5206 and PAN6264, only light lesions and infection appeared on the husk and kernel after inoculation. In comparison, in susceptible cultivars, symptoms on the surface of the husk appeared on day 2 or 3 after inoculation with conidial suspension of the U2 strain of S. maydis, indicative of a quick, successful penetration, with colonization of husks by the fungus. The kernels of susceptible cultivars were also infected severely by heavily ramified inter- and intracellular hyphae in the endosperm tissues (Fig. 2A and 2B). Furthermore, typical two-celled conidia were found individually in cells of endosperm tissue (Fig. 2C) 4 to 6 weeks after inoculation. These two-celled spores resembled morphologically those produced on synthetic media, as reported by Murphy et al. (30), except for a large amount of vacuoles. Apart from lytic function, vacuoles serve in storing ions and amino acids for protein synthesis (17). In addition, vacuoles might also be very...
important for fungi to synthesize enzymes needed for a successful colonization, and to moderate the osmotic pressure from plant if the host plant cells contain large quantity of vacuoles (9, 17).

Overall, infection progressed in the order of husk, endosperm, and then kernel, as revealed by both visual examination of symptoms and microscopic observation of mycelia under artificial inoculation conditions in this study, which was inconsistent with previous reports that under natural conditions, infection occurred in the order of the base of the ear, the sclerenchyma–placentae–embryos, and then the whole kernel (6, 8, 13).

**Enzymatic activities during host–pathogen interaction of S. maydis.** Cell wall degradation surrounding the penetrating hyphae was clearly visible, implying enzymatic involvement (Fig. 3A) in embryonic tissues of susceptible cultivars. Cell wall degradation resulted further in lacunae on the surface of endosperm cells and disruption of cell organelles in these tissues (Fig. 3B and 3C). However, callose-like deposition surrounding fungal hyphae was observed clearly in embryonic tissues of resistant, but not susceptible, cultivars (Fig. 3C). Callose was reported to act as a barrier against fungal colonization (6). Murphy et al. (29) described callose as a mucous layer that protects hyphae against dehydration. Plants defend against fungal attack (28) by physical strengthening of the cell wall via lignification (36), suberization and callose deposition (6), accumulation of hydroxyproline-rich glycoprotein (26), synthesis of...
antimicrobial compounds such as phytoalexins, and production of a variety of pathogenesis-related proteins (24). More recently, it was reported that *S. maydis* could secrete a special protein, Stm-cmp, to modify specifically the ChitA-F protein alloform, which is encoded by a known allele of the *chiA* gene in the strongly susceptible B73 line (32). Further investigation is needed as to whether the callose depositions observed in this study are directly associated with the interaction between Stm-cmp and targeted plant chitinase (32). More recently, Naumann and Wicklow (32) demonstrated that the susceptibility to modification of a fungal-targeted plant chitinase differs among inbred lines, suggesting that the LH82 and B73 lines’ alleles may be specific genetic determinants for resistance or susceptibility.

**Preferential infection of embryonic tissues and composition of pycnidia.** In resistant cultivars RS5206 and PAN6264, the endosperm was slightly affected by the pathogen, and no severe damage occurred after artificial inoculation. However, in the susceptible cultivar, endosperms were severely damaged at later stages (Fig. 3B and 3C). *S. maydis* favored colonization of the embryonic tissues by formation of pycnidia once maize kernels were infected (Fig. 4 A and 4B) (5). In pycnidia, spores appeared different morphologically, and degraded organelles of host cells mingled often (Fig. 4C). As colonization progressed, pycnidia formed in the kernels, especially in the embryos. The preferential colonization of embryonic tissue by *S. maydis* could be ascribed to its richness in nitrogen and other nutrients, rather than to those nutrients of the endosperm. Although starch is the primary component of the endosperm, the embryo also contains significant amounts. In a previous report, Flett and Wehner (16) indicated that *S. maydis* in maize needs additional nutrition to enhance its sporulation. Guo et al. (18) observed that the antifungal, ribosome-inactivating protein is located mainly in the endosperm, whereas zeamatin is located mainly in the embryo, which uniquely protects kernels from pathogen attack. The presence of abundant mycelia and pycnidia in the embryonic tissue could imply that *S. maydis* might be insensitive to zeamatin.

The morphologically different propagules and resting structures of *S. maydis* in maize along with its saprophytic nature (14) are likely associated with long-term survival of the pathogen in the field. This could explain the difficulty in controlling this disease exclusively by tillage or crop rotation (12, 15).

Diplodia toxin might be secreted at the tip of hyphae during infection and colonization in a way similar to that of the *Fusarium* toxin produced in wheat (21). The infected seeds could pose a long-term threat as food or forage for humans and domestic animals by diplodia toxin, a toxin known for its carcinogenic and neurotoxic effects (25, 33).
REFERENCES
