Fungal Morphogenesis Tracking of *Blumeria graminis* f. sp. *tritici* on Leaf Freed of Epicuticular Wax Using Scanning Electron Microscopy

Samar Mohamed Esmail, Ibrahim Sobhy Draz

Wheat Disease Research Department, Plant Pathology Research Institute, Agricultural Research Centre, Giza, Egypt

Email address:
dr.ibrahim_draz@yahoo.com (I. S. Draz)

Abstract: Fungal morphogenesis development of *Blumeria graminis* f. sp. *tritici* was tracked on leaves freed of epicuticular wax using the scanning electron microscopy during successive times, 1 day post inoculation (dpi), 2 dpi, 3 dpi, 4 dpi, 5 dpi and 7 dpi. A conidium seen 1 dpi landing on a leaf showed spore germination and the presence of primary germ tube and the appressria formation revealing their dimensions. Appressorial germ tube has elongated and swollen to form an infection structure, the appressorium has a hooked apical lobe. At 2 dpi, the network of tubular cells forms the mycelial hyphae growing over the leaf surface being fed by a haustorium hidden inside the cell under the appresorium. By 3 dpi, colony consisted of mycelial hyphae with rare hyphal lobes. Appressorial lobes tightly adhered to the surface of epidermal cells. At 4 dpi, extensive hyphal growth and repeated penetration from hyphal appressoria resulted in the formation of further haustoria and bulbous conidiophores. On 5 dpi, bulbous conidiophores have started generating conidia. By 7 dpi, well-developed fungal colony were formed with many chains of conidia sticking up into the air and can be wind spread to initiate new infection cycles. We cannot be sure that removing of the waxes did not affect the pathogen's ability to produce conidial exudates or extracellular material from its germ tubes, appressoria, or hyphae. However, we believe this is unlikely, since removing of leaf waxes prior to inoculation has very little effect on many different aspects of fungal development.

Keywords: Wheat Powdery Mildew, *Blumeria graminis*, Fungal Morphogenesis, Removal Epicuticular Wax, SEM Examination

1. Introduction

Powdery mildew fungus *Blumeria graminis* DC. E.O. Speer f. sp. *tritici* Em. Marchal (syn. *Erysiphe graminis* DC) is an obligate biotrophic pathogen that cause destructive foliar disease of wheat plant. The fungus *B. graminis* f. sp. *tritici* (*Bgt*) only infects epidermal cells and colonizes the host surface. The *Bgt* infection processes and the early interaction have been well-documented [1, 2]. It is generally accepted that the pathogen firmly attaches to the host surface by release of extracellular materials from fungal spores and germlings. Extracellular materials-mediated adhesion to the host surface has been reported by Carver et al. [3, 4], Wright et al. [5] and Zhang et al. [2]. The surface detail of biological material of the causal fungus *Bgt*, can be preserved to study by scanning electron microscopy (SEM). This approach has

been used in several studies of the obligate biotrophic fungus *B. graminis* DC. ex Merat, which causes powdery mildew of cereals and other Gramineae. For example, in an early study, Staub et al. [6] used cryofixation to preserve epicuticular leaf wax morphology and allow the resolution of tracks left in the waxes by fungal germ tubes that had been removed using gelatine film. They assumed leaf wax beneath germ tubes had been degraded by the fungus, but this remains unproved. Low temperature SEM studies also showed subtle changes in the surface morphology of *E. graminis* conidia within 30 min of their deposition; these changes are thought to be related to the release of conidial exudates containing esterase enzymes including cutinase [7, 8]. When these exudates were applied to barley leaf surfaces, LTSEM revealed that they appeared

to remove amorphous material, possibly cutin, interspersed between the crystalline epicuticular wax plates, although the wax plates themselves appeared unaffected [9]. These and other SEM studies of *Blumeria graminis* have concentrated on particular aspects of the host-pathogen interaction, and a general study of the fungal life cycle has not been undertaken. One of our current objectives was to perform such a study. The main purposes of the current paper were to determine the development of fungal morphogenesis, and to determine when and where they are formed. We decided to remove epicuticular leaf wax before inoculation to rule out the possibility that fungal morphogenesis were wax fragments. Epicuticular leaf wax may be removed using a glue made from cellulose acetate [10]; this exposes the smooth surface of the underlying cuticle proper consisting of cutin and embedded intracuticular wax [11]. Carver and Thomas [12], using this technique, showed that formation of normal, functional appreswax. They also confirmed that epicuticular wax removal caused no apparent damage to leaves and had no effect on their functional life span, and that, once removed, epicuticular waxes were not regenerated. In view of this, we felt justified in using leaves freed of epicuticular wax to study fungal morphogenesis formation, as well as for the general study of fungal development.

A further objective of the work reported here was to understand whether *B. graminis* f. sp. tritici produces extracellular materials that may be involved in adhesion of the fungus to the host surface. It has been proposed that attachment of spores and germ tubes is an essential prerequisite to infection by *Bgt* and other fungi. Therefore, there has been increasing interest in the means by which fungi adhere to their host [13–15]. For *B. graminis*, the process of adhesion remains poorly understood, although it may be that the early release of esterases, including cutinase, is related to adhesion [16, 17]. But while certain fungi produce substantial quantities of extracellular adhesive material that is relatively easy to visualize in association with spores, germ tubes, appressoria, or hyphae, such materials have not been seen in association with *B. gratninis* on host leaves. Epicuticular plate waxes on host leaves obscure details of the host fungus interface and may have hidden extracellular material. Our current use of leaves freed of epicuticular wax gave us the opportunity to search for evidence of extracellular fungal secretions that might otherwise be obscured by wax crystals.

2. Materials and Methods

2.1. Isolation of the Causal Fungus

Isolation process was done at the greenhouse of Wheat Disease Research Department, Sakha Agricultural Research Station, Kafr-Elsheikh, Egypt. To isolate *B. graminis* f. sp. *tritici*, typical powdery mildew-infected leaves collected from commercial wheat fields of Egyptian Governorates were picked and inoculated on 8-day old seedlings of highly sensitive cultivar (Sakha-61) according to the method described by El-Salamony [18] and Opalski et al. [19]. The inoculated seedlings were incubated in a dark dew chamber for 24 hrs at 20±2°C, 70-90% relative humidity and then moved to greenhouse-conditioned under 16 h light: 8 h dark photoperiod with light intensity of approximately 14000 luxmeter.

2.2. Removing of Leaf Epicuticular Waxes

Before inoculation, epicuticular leaf waxes were removed using the method of Whitehouse et al. [10], as followed by Carver and Thomas [12]. Briefly, the adaxial (upper) surface of seedling leaves was painted with a viscous solution of cellulose acetate dissolved in acetone. As the solvent evaporated (1-5 min), the epicuticular waxes became embedded in the cellulose acetate film and were removed when the film was peeled away. This revealed the smooth, almost featureless, surface of the cuticle proper.

2.3. Inoculation Process for SEM Examination

Ten-day old cultures of the pure isolates were used for inoculation the tested seedling leaves concerned for scanning electron microscope (SEM) examination. Three leaves bearing raised powdery mildew colonies were gently removed from the target culture and then shaken over the tested seedlings leaves. A microscope slide placed among the leaves was used to monitor inoculum density, which was standardized at 30-50 conidia/mm^{-2}. To track the development of the fungal morphogenesis using SEM, five specimens were taken and examined during successive times *i.e.* 1 day post inoculation (dpi), 2 dpi, 3 dpi, 4 dpi and 7 dpi. Sample preparation for SEM examination was carried out as described by Harley and Fergusen [20]. The examination and photographing were done through a Jeol scanning electron microscope (T.330 A) at the Central Laboratory of Agriculture Faculty, Ain Shams University, Cairo, Egypt.

3. Results

Fungal morphogenesis development of *B. graminis* f. sp. *tritici* was tracked using the scanning electron microscopy during successive times, 1 dpi, 2 dpi, 3 dpi, 4 dpi, 5 dpi and 7 dpi. On 1 dpi, the primary infection structures established tight adhesion contacts with the epidermal cell surface and numerous small fungal colonies were formed. A conidium seen 1 dpi landing on a leaf showed spore germination and the presence of primary germ tube (PGT) (3.72μm) and the appressria formation revealing their dimensions. Appressorial germ tube (4.32μm) has elongated and swollen to form an infection structure, the appressorium (32.6μm) has a hooked apical lobe (Figure 1).
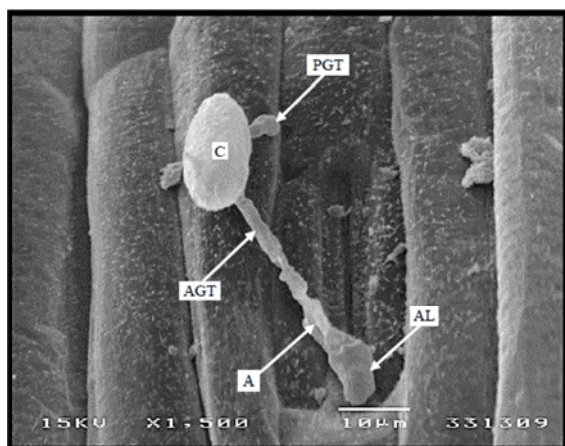
Figure 1. *Conidial development of Blumeria graminis f. sp. tritici on leaf epidermis. A conidium (C) seen 1 day post inoculation (dpi) landing on a leaf surface. The primary germ tube (PGT) (3.72μm) has emerged while appressorial germ tube (AGT) (4.32μm) has elongated and swollen to form an infection structure, the appressorium (A) (32.6μm) has a hooked apical lobe (AL).*

At 2 dpi, the network of tubular cells forms the mycelial hyphae (MH) (92.8μm) growing over the leaf surface being fed by a haustorium hidden inside the cell under the appresorium (43.4 μm) (Figure 2). By 3 dpi, colony consisted of mycelial hyphae with rare hyphal lobes; the hyphae attached to each other and were mostly arranged along the anticlinal walls of the epidermal cells. The appressorial lobes tightly adhered to the surface of epidermal cells (Figure 3).
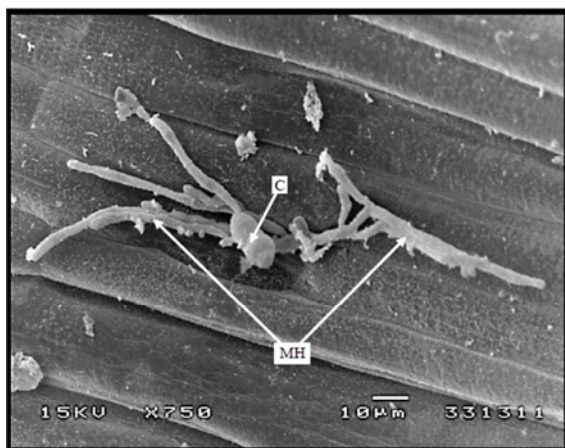

Figure 2. *Hyphal development of Blumeria graminis f. sp. tritici 2 dpi on leaf epidermis. The network of tubular cells forms the mycelial hyphae (MH) (92.8μm) growing over the leaf surface being fed by a haustorium hidden inside the cell under the appresorium (43.4 μm).*

At 4 dpi, extensive hyphal growth and repeated penetration from hyphal appressoria resulted in the formation of further haustoria (not shown) and bulbous conidiophores (11.9×5.12μm) (Figure 4). On 5 dpi, bulbous conidiophores have started generating conidia (Figure 5). By 7 dpi, well-developed fungal colony were formed with many chains of conidia sticking up into the air and can be wind spread to initiate new infection cycles. The colony was with tightly interlaced hyphae whose lobes had tight adhesion contact
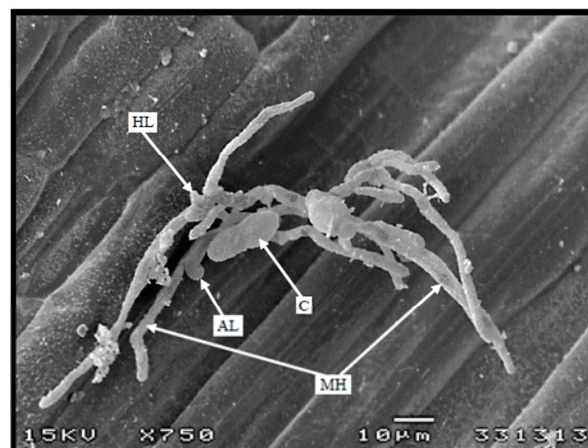
with the epidermal cells (Figure 6).

Figure 3. *Hyphal development of Blumeria graminis f. sp. tritici 3 dpi on leaf epidermis. Colony consisted of mycelial hyphae (MH) with rare hyphal lobes (HL). The hyphae attached to each other and were mostly arranged along the anticlinal walls of the epidermal cells. The appressorial lobes tightly adhered to the surface of epidermal cells.*

Figure 4. *Morphogenesis development of Blumeria graminis f. sp. tritici 4 dpi on leaf epidermis. Extensive hyphal growth and hyphal appressoria were first seen. Penetration from hyphal appressoria resulted in the formation of further haustoria (not shown) and bulbous conidiophores (BC) (11.9×5.12μm).*

Figure 5. *Conidiophore development of Blumeria graminis f. sp. tritici 5 dpi on leaf epidermis. The formed bulbous conidiophores (11.9×5.12μm) have started generating chain of conidia (GC).*

Figure 6. Complete fungal morphogenesis of Blumeria graminis f. sp. tritici 7 dpi on leaf epidermis. A well-developed fungal colony were formed with many chains of conidia (CC) sticking up into the air. AC; Apical conidium (6.4×12.6µm) and SAC; Sub-apical conidium (5.9× 11.4µm).

4. Discussion

The surface detail of biological material of the causal fungus *B. graminis* f. sp. *tritici* (*Bgt*), can be preserved for study by scanning electron microscopy (SEM). This approach has been used in several studies of the obligate biotrophic fungus *B. graminis* DC. ex Merat, which causes powdery mildew of cereals and other Gramineae. Following contact the ectoparasitic obligate biotrophic fungus *Bgt* with the host surfaces, the conidia form a primary germ tube (PGT) and an appressorial germ tube approximately 0.5–2 and 4–8 h after inoculation, respectively. The appressorial germ tube begins to elongate and after 9–12 h differentiates a lobed appressorium. A peg forms under this appressorium, which penetrates the host cell wall and establishes a digitate haustorium within an epidermal cell. Papilla formation occurs in the leaf epidermal cell subjacent to the germ tubes. This local response in the outer epidermal cell wall excludes or delays a significant proportion of the attempted penetrations by the fungus [21]. Successful establishment of a haustorium, the only fungal organ that invades the host, is followed by the formation of secondary hyphae from the appressorium. An elongating secondary hypha is the starting point for the development of a fungal colony. From the epicuticular hyphae, secondary appressoria are formed and, from these, secondary haustoria are established in epidermal cells. About 3–4 days after the primary infection, conidiophores are formed on the hyphae, sporulation starts and spores can be wind spread to initiate new infection cycles [22].

In this study, fungal morphogenesis development of *B. graminis* f. sp. *tritici* was tracked on leaves freed of epicuticular wax using the scanning electron microscopy during successive times, 1 dpi, 2 dpi, 3 dpi, 4 dpi, 5 dpi and 7 dpi. On 1 dpi, the primary infection structures established tight adhesion contacts with the epidermal cell surface and numerous small fungal colonies were formed. A conidium

seen 1 dpi landing on a leaf showed spore germination and the presence of primary germ tube and the appressria formation revealing their dimensions. Appressorial germ tube has elongated and swollen to form an infection structure, the appressorium has a hooked apical lobe. The conidial germination patterns *i.e.* the site of emergence of germ tube from conidia body and the morphology of appressorial germ tube, producing two types of germ tubes, primary tubes, which never penetrate the host cells, and longer thicker secondary tubes, which produce penetration hyphae [23]. Recently, the body of the germinated conidia has been recognized to be served as a site for conidiogenesis along with the hyphae [24, 25]. At 2 dpi, the network of tubular cells forms the mycelial hyphae growing over the leaf surface being fed by a haustorium hidden inside the cell under the appresorium. By 3 dpi, colony consisted of mycelial hyphae with rare hyphal lobes; the hyphae attached to each other and were mostly arranged along the anticlinal walls of the epidermal cells. The appressoria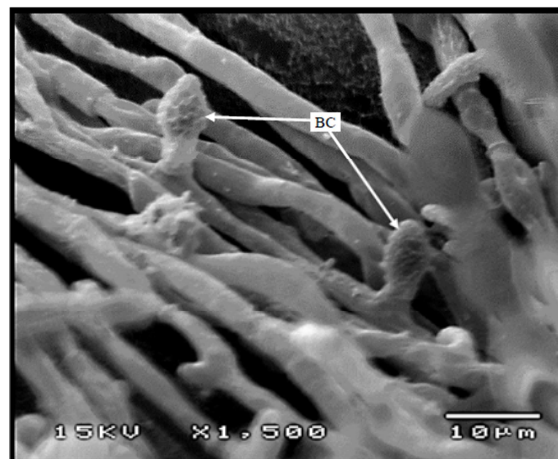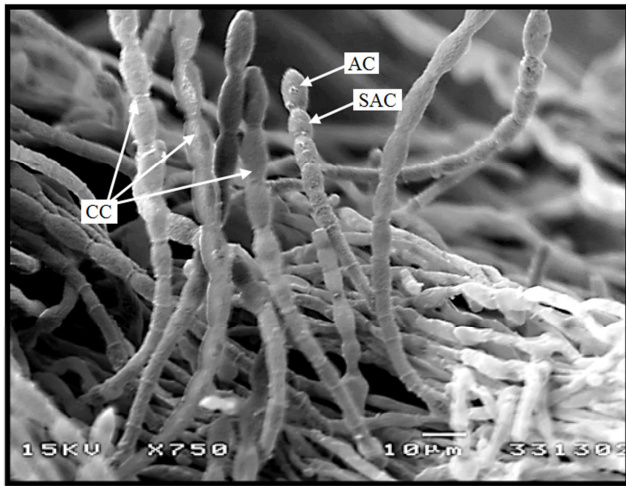l lobes tightly adhered to the surface of epidermal cells. At 4 dpi, extensive hyphal growth and repeated penetration from hyphal appressoria resulted in the formation of further haustoria (not shown) and bulbous conidiophores. On 5 dpi, bulbous conidiophores have started generating conidia. By 7 dpi, well-developed fungal colony were formed with many chains of conidia sticking up into the air and can be wind spread to initiate new infection cycles. The colony was with tightly interlaced hyphae whose lobes had tight adhesion contact with the epidermal cells. Basically, complete infection structures were formed only on 3 dpi, also a high degree of adhesion of the appressorial lobes to the plant epidermal cells was observed [26].

Where a much older (2 dpi) PGTs had been displaced, the PGT was so strongly stuck to the host cuticle that a piece of cuticle was torn away from the leaf surface and remained attached to the displaced PGT tip. This suggests that the extracellular material acts as an adhesive between the PGT and host surface [27]. Whether attempted infection by first appressorial lobes had failed or succeeded could not be determined until either a second appressorial lobe had formed indicating failure, or hyphae developed indicating successful infection [26, 28]. Both successful and unsuccessful first appressorial lobes could thus be recognized at 1 dpi, but there were no obvious differences between these lobes types in terms of the appearance, position, or number present on the appressorial lobe. By the time, second appressorial lobes were first seen (1 dpi), they were present on their surface.

Colonies at 7 days had commenced sporulation and conidiophores and chains of conidia at all stages of development could be examined. Although sporulation seen by SEM has been described previously and conidiogenesis was described in detail [29, 30]. Present SEM revealed details of surface structures not previously seen. As Cole and Samson [30] described, a septum then formed just above the conidiophore apex; in one case, this septum was clearly revealed by an accidental breakage. Additional septa developed in acropetal succession as individual conidia matured. In some of micro-graphs, the septa1 region between

developing conidia within a chain appeared as a ridge like ring around the fertile hypha. We never saw this ridging and conclude that it is an artefact of the procedures used for these SEM studies. As conidia approached maturity, their surface became covered in the characteristic protrusions that we could not distinguish from. By contrast, the meristematic zone of Cole and Samson [30] of the fertile hypha tended to remain smooth during its elongation phase.

Adhesion to host surfaces represents an initial strategy for successful infection by many phytopathogenic fungi [31, 32]. Furthermore, components of the plant surface or cuticle and their breakdown products can be taken up by the pathogen as part of a signalling system triggering spore germination and infection structure differentiation [17, 33–35]. It is generally accepted that the pathogen firmly attaches to the host surface by release of extracellular materials (ECM) from fungal spores and germlings. ECM-mediated adhesion occurs in a wide range of pathogenic fungi, including the rice blast *Magnaporthe grisea* [36], the anthracnose *Colletotrichum* spp. [37,38], the rusts *Puccinia* and *Uromyces* spp. [39] and the powdery mildews *Uncinuliella australiana* [40], *Erysiphe pisi* [3,32], and *Blumeria graminis* [2–5]. Hydrophobic epicuticular waxes minimize the adhesion of water and other particles on the surface of leaves, thereby keeping leaves clean and enhancing their ability to trap light for photosynthesis [41].

Adhesives of the powdery mildew fungi *B. graminis* and *U. viciae-fabae* contain nonspecific esterases and cutinases [16, 17, 42], and the isolated *B. graminis* ECM is able to degrade host surface features [7], suggesting that lipolytic enzymes such as esterases and cutinases are released into the fungal ECM and lead to adhesion [43]. Distinction between these enzymes has been proposed based on their substrate specificity and their capacity to hydrolyze esters in solution or emulsion. Esterases act on solutions of short-chain fatty acyl esters, whereas lipases have the capacity to act on water-insoluble long-chain fatty acyl groups. Cutinases, considered a link between esterases and lipases for substrate preference [44], are generally small enzymes of approximately 22 to 25 kDa [45, 46] that are active in both soluble-phase and emulsified interface and do not show so-called interfacial activation, a phenomenon commonly observed in lipases [47]. Although cutinases are also able to efficiently hydrolyze triglycerides [48], they exhibit a higher specificity for short-chain acyl groups, and this feature has been used as an indicator of cutinase activity.

Evidence [7–9, 16, 17], including SEM studies, shows that *B. grarninis* f. sp. *hordei* conidia release exudates and enzymes including cutinase onto the leaf surface during the first 30 min after inoculation. However, we saw no sign of either exudates or of host surface degradation in the area surrounding conidia in situ, or in the locality from which conidia had been removed with gelatine. Unlike of Feng et al. [49], pre-treatment of wheat leaves with *Lip1*, thereby removing leaf surface wax, severely compromises components of fungal pathogenicity, including conidial adhesion, appressorium formation, and secondary hypha

growth. This suggests that *Lip1* activity releases cues from the host surface to promote pathogen development and infection.

There are several possible explanations. The host cell recognizes and quickly responds to PGT and appressorium contact, with localized accumulation of autofluorogenic phenolic material [50–52], redirection of cytoplasmic streaming [53, 54], cytoplasmic aggregation [55, 56], elevation of phenylalanine ammonia lyase (PAL) activity [57], and increased expression of various host response genes [55], and yet no one has reported a host cellular response that is definitely attributable solely to contact with conidia. Thus any effects due to secretions from the spore are too slight to initiate a detectable host cell response. Apparently, conidial exudates have subtle effects. These may include altering the host surface in the area of the infection court enabling or facilitating adhesion [14], possibly by increasing the hydrophilicity of the host surface [8].

Previous studies of Ryabchenko et al. [58] have shown that powdery mildew morphogenesis in the course of pathological process, from conidia germination to the formation of sporulating colonies, largely depends on fungus compatibility with the host plant. The adhesive interactions between the infection structures (appressorial and hyphal lobes) and the surface of epidermal cells are important for the development of the pathogen colonies. The lobes of appressoria and the hyphal lobes are the maternal cells of haustoria. Their number in the active physiological state defines the size and formation pattern of the colonies. The envelopes of the appressoria and hyphal lobes are denser and rougher than the germ tubes and hyphae. This facilitates their tight adhesion to the affected plant surface and provides for their longer survival of under unfavorable conditions. Both these factors are important for parasitic adaptation of the pathogen. In the case of a compatible combination, both conidia and appressoria retain their turgor for a long time, which indicates their active involvement in vital activity of the pathogen at the early stages of its interaction with the host plant. The developing colonies consist of tightly interlaced hyphae, usually, with a small number of hyphal lobes [26]. In the case of low compatibility, the number of hyphal lobes increases, but their adhesive interaction with the plant tissue surface weakens, provoking the pathogen to search for other penetration sites. In this case, the fungal colonies are formed of loose hyphae arranged both along and across the anticlinal walls.

Staub et al. [6] interpreted "tracks" left in barley leaf surface waxes following the removal of *B. graminis* germlings as evidence that leaf waxes had been degraded by the fungus. While this may be true, our current observations offer an alternative explanation. It may have been that removing of the fungus simply exposed a track of extracellular material that had filled the spaces between epicuticular wax plates creating an appearance of surface erosion. Alternatively, the waxes may have adhered to the fungus and been removed with it. At present it is not clear which of the possible explanations is correct, although proof that the fungus produces wax degrading enzymes is lacking.

Indeed, although Kunoh et al. [9] showed that conidia release a substance capable of removing, presumably through degradation, amorphous material (probably polymeric cutin) interspersed between wax plates on the leaf surface, the wax plates themselves did not appear to be eroded. It has been proposed that attachment is an essential prerequisite to successful infection by fungal pathogens including *B. gratninis* [7, 14]. Mustafa et al. [59] found that systemic resistance was associated with a significant reduction of *B. graminis* haustorium formation in epidermal leaf cells of mycorrhizal wheat and an accumulation of phenolic compounds and H_2O_2 at *B. graminis* penetration sites. Moreover, gene expression analysis demonstrated upregulation of genes encoding for several defence markers, such as peroxidase, phenylalanine ammonia lyase, chitinase 1 and nonexpressor of pathogenesis-related proteins 1 in mycorrhizal wheat only in the absence of the pathogen.

5. Conclusion

We cannot be sure that removing of the waxes did not affect the pathogen's ability to produce conidial exudates or extracellular material from its germ tubes, appressoria, or hyphae. However, we believe this is unlikely, since removing of leaf waxes prior to inoculation has very little effect on many different aspects of *B. graminis* development [26]. Thus, our current evidence showed that the time course of germling development on wax-free leaves was similar to that reported by others using intact leaves [27]. Additionally, previous studies of Carver and Thomas [12] showed little effect of leaf wax removal on *B. graminis* germination rates, the developmental characteristics of germlings, or the ability of appressoria to form haustoria. Through understanding the nature of any process essential to establishment of pathogenicity, we gain insight with potential value for the design of host resistance characteristics or chemical fungicides to control the disease.

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