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Structural characterisation of the interaction between *Triticum aestivum* and the dothideomycete pathogen *Stagonospora nodorum*

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Abstract

The interaction between *Stagonospora nodorum* and a susceptible wheat cultivar was investigated using a range of microscopic techniques. Germination of pycnidiospores occurred approximately 3 h after making contact with the leaf surface and was followed by attempted penetration 8–12 h later. Penetration was observed through stomata and also directly through periclinal and anticlinal epidermal cell walls. Penetration down the anticlinal cell walls appeared to occur without a differentiated penetrating structure whilst structures identified as either lateral appressoria or hyphopodia were typically present when penetrating over a periclinal cell wall. Once inside the leaf, the fungus continued to grow for the next 4–5 days colonising all parts of the leaf except the vascular bundles. Only in the later phase of the infection was total host cell collapse apparent. Evidence of polyphenolic compounds was observed. The infection cycle was completed within 7 days as indicated by sporulation on the leaf surface. These results have allowed us to understand how the fungus physically interacts with the leaf and will help the overall understanding of the infection process.

Introduction

Phaeosphaeria nodorum (anamorph *Stagonospora nodorum*) (Bathgate and Loughman, 2001) is a necrotrophic dothideomycete phytopathogen that is the causal agent of leaf and glume blotch on wheat and is responsible for $60M (AUD) of crop loss in Australia each year (Brennan and Murray, 1998).

Detailed molecular studies are currently underway to determine the genetic basis of how *S. nodorum* infects wheat (Solomon et al., 2003, 2004a, b; 2005; Solomon and Oliver, 2004). However to understand how the pathogen interacts with its host at a molecular level, there must be understanding of the physical interaction at the cell and tissue level. Previous reports investigating the cytology of the interaction have been useful in determining facets of the infection such as the timing of the infection process and percentage of successful penetration attempts (Baker and Smith, 1978; Bird and Ride, 1981; O'Reilly and Downes, 1986; Zinkernagel et al., 1988; Cunfer, 1999). However detailed descriptions of the mode of penetration and the nature of the penetrating structure have either been lacking or conflicting. For example, there are some previous studies which have observed penetration through stomata (Straley and Scharen, 1979; O'Reilly and Downes, 1986) whilst others have not (Shipton et al., 1971; Baker and Smith, 1978). To truly understand the interaction, these facets of the disease need to be clarified.

Materials and methods

Stagonospora nodorum SN15 was provided by the Department of Agriculture, Western Australia.

All media and growth conditions were as previously described (Solomon et al., 2004a). The preparation and transformation of *S. nodorum* protoplasts were also as described (Solomon et al., 2003). Green fluorescent protein (GFP) expression in *S. nodorum* was achieved by transforming protoplasts with pGpdGFP (Sexton and Howlett, 2001).

Infections of *S. nodorum* on wheat leaves were undertaken on detached leaves from susceptible 2 week-old wheat seedlings (cv. Amery) as previously described (Solomon et al., 2003). All subsequent microscopic analyses were performed on at least three biological replicates and images shown were typical of what was observed.

Lesions on wheat leaves caused by *S. nodorum* were examined by light microscopy. Diseased leaf tissue was stained and cleared with trypan blue using a method modified from that described by Shipton and Brown (1962) and Bruzzese and Hasan (1983). The presence of callose was stained as previously described (Adam and Somerville, 1996). For examination of the infection using scanning electron microscopy (SEM), lesions were excised from the detached wheat leaf and fixed overnight in 25 mM phosphate buffer containing 2.5% glutaraldehyde. This was then rinsed once and fixed in phosphate buffer containing 2% osmium tetroxide at room temperature. The fixed tissue was dehydrated in 30, 50, 75, 95, and 100% ethanol washings then transferred to an ethanol/amyl acetate mixture and further washed by two rounds of 100% amyl acetate. The tissue was then dried with a critical point dryer, mounted, sputter coated with gold, and viewed with a Philips XL Series SEM using an accelerating voltage of 5.00 kV.

Confocal laser scanning microscopy was performed using a LaserSharp Confocal System (BioRad) combined with a Nikon inverted microscope. GFP images were collected by exciting at 488 nm and collecting at both 522 and 605 nm. Optical sections were typically collected over a depth of 40 μm using 1 μm sections. Hand-cut sections were prepared essentially as described by O'Brien and McCully (1981).

Results and discussion

Germination of the pycnidiospores, observed using trypan blue stained samples, occurred within 3–12 h of making contact with the leaf surface (Figure 1a). Spore germination was typically unipolar although bipolar germination was occasionally observed. After germination, hyphae were observed to grow over the surface of the leaf. There was no evidence that the growth was directed or thigmotropic. As with all fungal phytopathogens, germination occurred in the absence of any external nutrient source and thus appeared reliant on intracellular energy reserves. It has previously been demonstrated that the catabolism of lipids is likely to be the carbon source for germination (Solomon et al., 2004a). However, these energy reserves are limited and for the fungus to continue growing, it must penetrate the leaf to gain access to the rich nutrient reserves within the leaf.

Within 12 h of germinating, penetration of the leaf surface was observed. The first evidence of penetration was observed through stomata (Figures 1b–c; 3a–b). Alteration of the hyphae such as swelling prior to penetration was neither evident nor was differentiation of any obvious penetrating structure observed. Penetration through stomata by *S. nodorum* has been previously reported, but there are also reports where stomatal entry was not observed (Shipton et al., 1971; Baker and Smith, 1978; O'Reilly and Downes, 1986). Our results conclusively demonstrate that at least this strain of *S. nodorum* is able to enter the leaf through stomata. The lack of hyphal differentiation upon reaching stomata is similar to what is observed in the tomato pathogen *Cladosporium fulvum* (De Wit, 1977). This is a practical method to penetrate the surface as the fungus does not have to utilise/mobilise large energy reserves as is the case for pathogens such as *Magnaporthe grisea* (Thines et al., 2000). Other pathogens, such as *Mycosphaerella graminicola*, also enter through stomata but tend to form appressorial-like structures at the opening (Duncan and Howard, 2000).

Within 18 h of germination, direct penetration of the leaf surface was observed over both periclinal and anticlinal epidermal cell walls (Figure 1d–i). In nearly all cases observed, no swelling or differentiation of the hypha was observed when penetration was attempted at anticlinal cell walls (Figure 1d and e). A similar mode of penetration has been previously reported for *Fusarium* species where penetration was found to be dependent on cell wall degrading enzymes and cutinases (Kolattukudy et al., 1995; Rodriguez-Galvez and Mendgen, 1995; Mendgen et al., 1996).

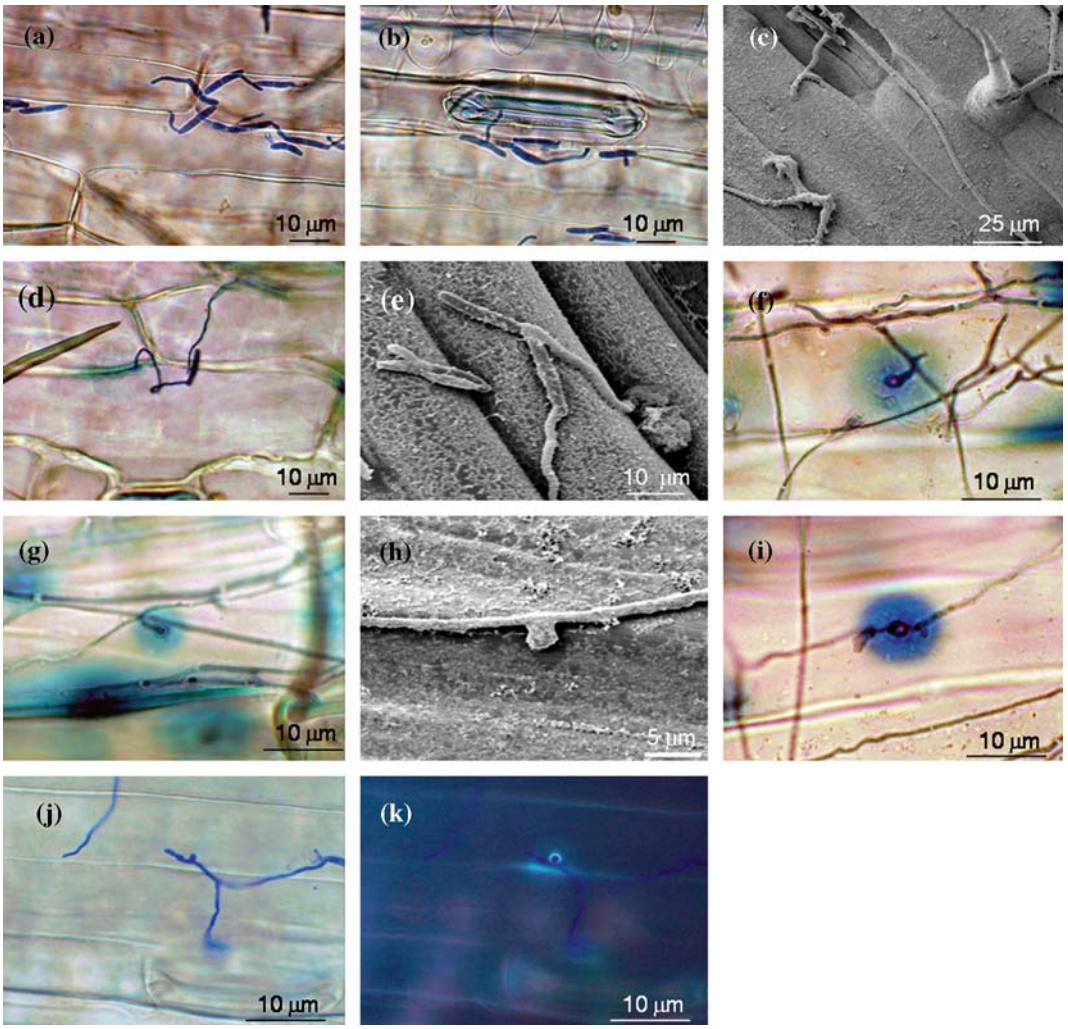

Figure 1. Microscopic images of *S. nodorum* on the leaf surface. (a) Germinating spores 3 h post-inoculation; (b–c) Germinating spores at 8 h post-inoculation showing hypha entering a stomate; (d–e) Direct hyphal penetration down anticlinal cell walls without differentiation of a penetrating structure; (f–h) Typical examples of sessile unlobed hyphopodia; (j) Example of intercalary unlobed hyphopodium; (j–k) Aniline blue stained sample captured using light microscopy (j) and fluorescence microscopy (excitation 365 nm) (k) demonstrating callose deposition under the penetrating structure; Note that due to the undulating nature of the wheat leaf surface, focusing problems prevented higher magnification images from being captured using light microscopy.

However when penetrating periclinal cell walls, and very occasionally anticlinal cell walls, hyphae appeared to differentiate two penetrating structures. The most commonly observed was a simple unlobed structure that was slightly swollen at the tip (Figure 1f–h). The second structure, which was rare in occurrence, appeared as a large swelling within the hypha itself (Figure 1i). Whilst some reports found no evidence of a penetrating structure in *S. nodorum*, other previous studies have observed that *S. nodorum* does produce appressoria (Shipton et al., 1971; Baker and Smith, 1978; O'Reilly and Downes, 1986). In contrast to previously described appressoria (Howard, 1997), the structures described in this study exhibited only moderate swelling and were not terminal implying that these were not appressoria. In an early review of appressoria, Emmett and Parbery (1975) broadly defined appressoria as structures whose basic function is to gain entry into a host. Within the review, varying classes of appressoria were defined with those termed lateral appressoria or hyphopodia

appearing most similar to the structures described in this study. A later review by Walker (1980) provided detailed descriptions and clear illustrations of varying types of hyphopodia. By comparing the structures observed within this study to those described by Walker (1980), *S. nodorum* appears to produce both sessile unlobed (Figure 1f–h) and unlobed intercalary hyphopodia (Figure 1i). It is clear from past literature that the terms of appressoria and hyphopodia have been interchangeable and either term could be used to describe the structures in this study. Given the obvious differences apparent between the structures in this study to the well characterised appressoria of *Magnaporthe grisea* and *Colletotrichum* species (Deising et al., 2000; Talbot, 2003), and the close similarity to previously described hyphopodia, the structures described in this study will be referred to as hyphopodia.

The best studied hyphopodia are those from the wheat root pathogen *Gaeumannomyces graminis* var. *graminis* (Walker, 1980; Walker, 1981; Money et al., 1998). Money et al. (1998) showed that while the hyphopodia from *G. graminis* are melanised and can withstand high turgor pressures, melanin synthesis itself is not required for pathogenicity of the fungus. It must also be noted that in contrast to the hyphopodia identified in this study, the hyphopodia described for *G. graminis* var. *graminis* are lobed and include examples such as the stalked lobed with stigmatocyst and the developing plate mycelium varieties of hyphopodia (Money et al., 1998). It is also interesting to note that *M. grisea* is able to use hyphopodia to infect rice roots rather than the appressorium mediated method of penetration used on leaves (Sesma and Osbourn, 2004). Structures very similar to those identified in this study have also been reported for the foliar pathogens *Erysiphe pisi* and *Ucinula necator* (Falloon et al., 1989; Heintz and Blaich, 1990). In highlighting the inconsistency with naming these structures, the authors of both papers reported the presence of appressoria. However a later review by Howard (1997) reported these structures as hyphopodia that were utilised to spread the fungus epiphytically once the infection had been established. Whilst a role in epiphytic growth cannot be excluded, a previous study investigating cAMP signal transduction in *S. nodorum* showed that the then unidentified structures appeared to play a role in penetration (Solomon et al., 2004b).

Also evident with most direct penetration attempts was evidence of callose deposition as demonstrated by aniline blue staining (Figure 1k). Callose is a complex carbohydrate normally associated with a defence response of the host. It is not clear though from the stained samples whether or not the direct penetration attempts themselves were successful. It has been previously reported that most of these penetration attempts fail with only 1–5% being successful (Bird and Ride, 1981). To confirm that the fungus was able to directly penetrate the surface other than through natural openings, susceptible leaves were infected with a strain of *S. nodorum* transformed with GFP for the first time and examined using confocal scanning laser microscopy (CSLM). At 2 days post-inoculation (Dpi), the GFP expressing hyphae were observed over much of the leaf surface and were seen to be penetrating through stomata (Figure 3a–b). Also apparent was a region of high fluorescent intensity (marked f on Figure 2a) at a region on the leaf clearly distinct from stomata. To determine whether this was a successful direct penetration attempt, a transverse section (marked with a white line in Figure 2a) was captured (Figure 2b.) The green fluorescence was clearly seen below the surface of the leaf and appeared to have reached the mesophyll layer confirming that the fungus was able to penetrate the leaf surface directly. To determine if either direct or stomatal penetration was the dominant route of entry into the leaf, 100 penetration events, using three biological replicates, were assessed. It was found that neither mode appeared dominant with 57 ± 15 of the 100 attempts being direct and 45 ± 28 occurring through natural openings. Also interesting is that previous studies have identified significant subcuticular growth of *S. nodorum* after breaching the cuticle (Zinkernagel et al., 1988). For the strain of *S. nodorum* and variety of wheat examined during the course of this study, there was little evidence of subcuticular growth.

To determine how *S. nodorum* grew once inside the leaf, transverse sections of leaves infected with *S. nodorum* SN15-GFP were examined by CSLM. Samples were collected by slicing vertically through the carrot and the leaf, and in doing so, collecting transverse sections of the infected leaves. These transverse sections collected over a 4 day period are shown in Figure 3. Typical stomatal penetrations are shown at 3 Dpi in Figure 3a–b whereby

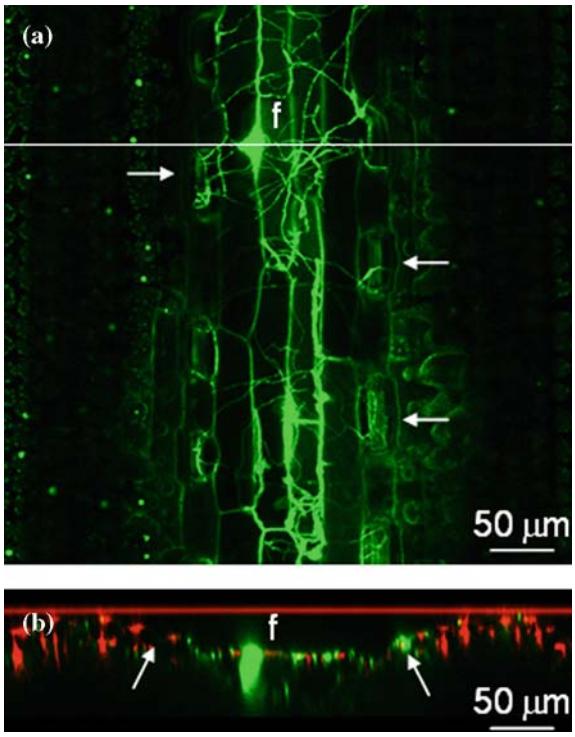

Figure 2. Confocal image of a leaf infected with *S. nodorum* SN15-GFP at 2 Dpi. The image captured was a z-series collected over 40 μm at 1 μm intervals. (a) A plan view looking on top of the leaf. Penetration via stomata is indicated by the white arrows. f denotes a region of high fluorescent intensity suggesting a possible direct penetration attempt. (b) A vertical cross-section of the image in (a) taken at the position of the white line.

S. nodorum had begun to proliferate throughout the mesophyll layer and indeed had nearly reached the distal side of the leaf. Yellow autofluorescence was also apparent indicating the presence of polyphenolic compounds. Cellular structure of the leaf appeared intact; however there were some indications of cellular collapse. By 4 Dpi, *S. nodorum* continued to grow within the leaf and although no evidence of sporulation was observed, yellow autofluorescence was still evident and cellular collapse was more apparent (Figure 3c). At 5 Dpi, the mesophyll layer had almost completely collapsed; the only apparent cellular structure remaining intact during this proliferation stage was that of the vascular bundle (Figure 3d and e). This is in agreement with earlier reports that the sclerenchyma tissue around the vascular bundles prevents infection of the vascular tissue by *S. nodorum* (Baker and Smith, 1978; Bird and Ride, 1981). This lack of colonisation also explains the absence of systemic infection, in contrast to the closely related canola pathogen, *Leptosphaeria maculans* (Hammond et al., 1985). Cellular structure within the leaf appeared to be largely intact at 3 Dpi, but by 6 Dpi, nearly all epidermal and mesophyll cells had collapsed. The reasons for this collapse are unclear; however it has been suggested that it might be due to secreted avirulence proteins, toxins or cell wall degrading enzymes (Bird and Ride, 1981; Essad and Bousquet, 1981; Bethenod et al., 1982; King et al., 1983; Bousquet and Kollmann, 1998). Recent studies have identified that *S. nodorum* does possess host-specific toxins (Liu et al., 2004a, b; Xu et al., 2004).

By 6 Dpi, the infection cycle neared completion and sporulation of the fungus was clearly apparent (Figure 3f–g). The infected leaf was typically completely chlorotic and necrotic and all cellular layers, including the vascular bundle had collapsed. Asexually formed pycnidia appear to form on both surfaces of the leaf. A closer examination of the pycnidium in Figure 3f revealed the green fluorescence to be localised within the pycnidia with only the subparietal layer and the conidiogenous cells fluorescing. The conidia did not appear to fluoresce. The structure of the pycnidia appear to be consistent with what has been previously observed in culture (Douaiher et al., 2004). As previously mentioned, conidia from *S. nodorum* SN15-GFP did not appear to fluoresce in mature pycnidia although the conidiogenous cells did. This was expected as the transcription of the GFP gene is driven by a *GpdA* promoter that is involved in glycolysis. It is likely that the conidiogenous cells involved in the synthesis of conidia are metabolically active and thus fluorescent. It is doubtful though that mature pycnidia would be undergoing glycolysis and consequently the absence of fluorescence is not surprising. These experiments do, however, provide a valuable insight into the metabolic compartmentalisation within pycnidia.

In conclusion, this study has clarified fundamental features of the interaction of *S. nodorum* and wheat. An understanding of these features will aid future studies in understanding how the fungus interacts with its host at a molecular level.

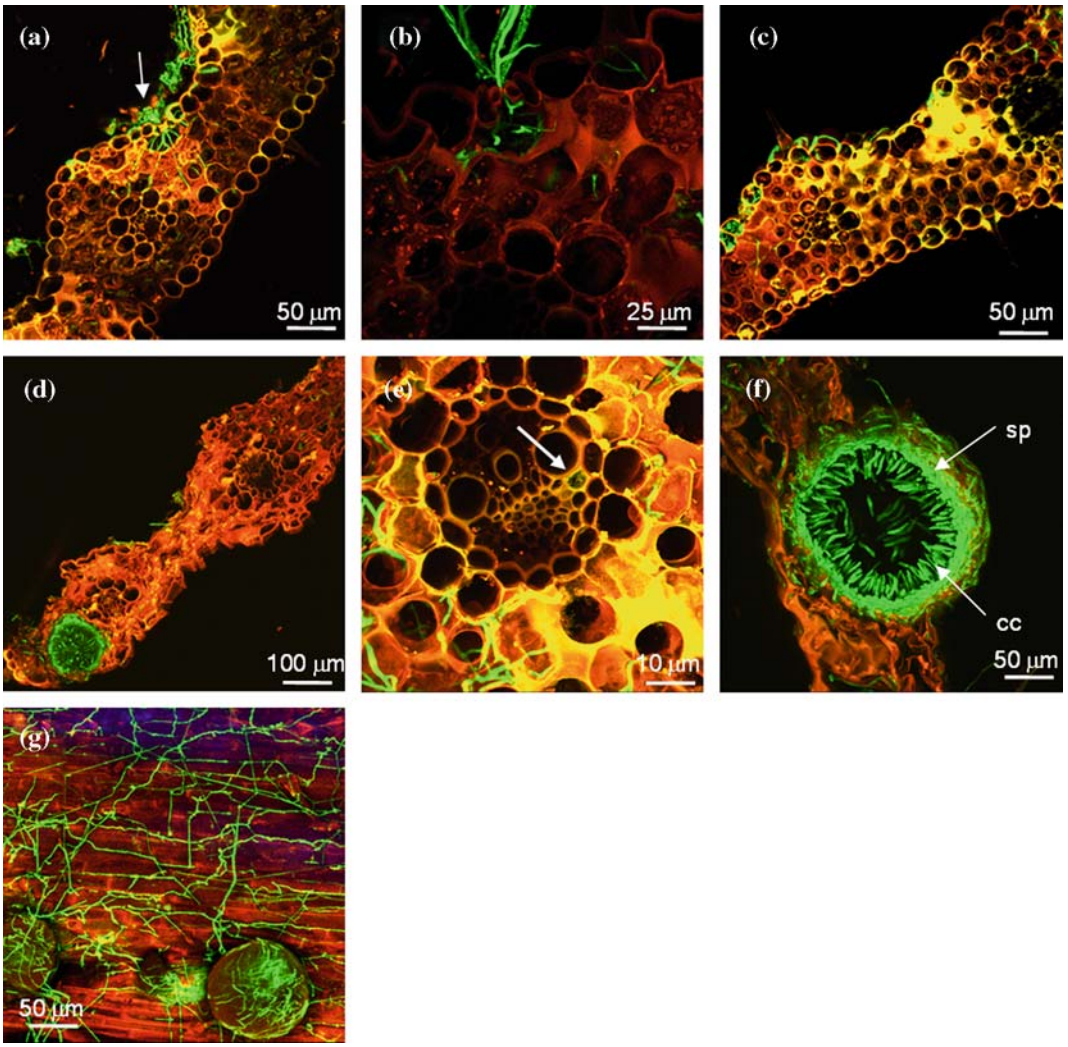

Figure 3. Confocal images of hand-cut transverse section of leaves infected with *S. nodorum* SN15-GFP. (a) 3 Dpi, penetration via stomata is indicated by the white arrow; (b) A higher magnification CSLM image demonstrating stomatal penetration at 3 Dpi; (c) 4 Dpi; (d) 5 Dpi, note the almost complete collapse of cellular structure and the onset of sporulation; (e) Image of vascular bundle at 5 Dpi. The white arrow highlights a potentially colonised cell within the vascular bundle. Colonisation and collapse of the vascular bundle is typical towards the end of the life cycle when the leaf is no longer viable; (f) Hand-cut transverse section detailing the structure of a pycnidium, sp – subparietal layer, cc – conidiogenous cells; (g) Plan view captured showing the pycnidia forming on the surface of the leaf at 6 Dpi.

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