

# Identification and localisation of glycoproteins in the extracellular matrices around germ-tubes and appressoria of *Colletotrichum* species

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A monoclonal antibody (MAb), UB31, is described that binds to the extracellular matrix (ECM) surrounding germ-tubes and appressoria, but not conidia, of the bean anthracnose fungus, *Colletotrichum lindemuthianum*. Comparative localisation studies with MAb UB26, which has the same cell type specificity, suggest that the ECM is heterogeneous in composition. Immunofluorescence showed that UB31 labelled appressoria more intensely than germ-tubes, whereas UB26 labelled these structures to a similar extent. Immunofluorescence and TEM-immunogold labelling showed that UB31 antigens were located close to the appressorial wall, while UB26 antigens extended further away from the wall. MAb UB31 bound to the ECMs of all six *Colletotrichum* species tested. Western blotting and ELISA indicated that the antibody recognises a high  $M_r$  glycoprotein (> 200 000) that may be linked to melanin. The glycoprotein recognised by UB31 was not removed from substrata by ultrasonication, suggesting it may contribute to germling adhesion.

## INTRODUCTION

The spores, germ-tubes and appressoria of most fungal plant pathogens are surrounded by mucilage, which may take the form of water-soluble fluids (Nicholson & Moraes 1980) or more rigid, polymerised materials (Kwon & Epstein 1997, Sugui, Kunoh & Nicholson 1999). Collectively, these mucilages are often referred to as extracellular matrices (ECMs), and in this context the term is usually taken to mean materials external to the polysaccharide-rich cell wall. There is increasing evidence that ECMs have several important functions during the pre-penetration development of fungal pathogens on plant surfaces. These include protection from desiccation and other environmental stresses (Moloshok *et al.* 1993, Nicholson & Moraes 1980), dispersal of secreted enzymes (Deising *et al.* 1992, Doss 1999), perception of host topographical signals (Epstein *et al.* 1987) and, in particular, adhesion of spores and infection structures to host surfaces (Braun & Howard 1994, Epstein & Nicholson 1997).

Among the anthracnose fungi (*Colletotrichum* species), different types of ECM mediate the adhesion of conidia, germ-tubes and appressoria. The initial at-

tachment of ungerminated conidia appears to involve hydrophobic interactions between substrata and protein components of the spore coat (Hughes *et al.* 1999, Mercure, Leite & Nicholson 1994, Sela-Buurlage, Epstein & Rodriguez 1991). This is a pre-formed ECM that forms a fibrillar layer overlying the cell wall and is composed largely of glycoproteins (Hughes *et al.* 1999). In addition, the conidia of some *Colletotrichum* species (e.g. *C. graminicola*, *C. gloeosporioides*, *C. destructivum* and *C. magna*) release a thin film of glycoprotein ECM at the spore-substratum interface (Jones, Bailey & O'Connell 1995, Mercure, Kunoh & Nicholson 1995, O'Connell *et al.* 2000), which may consolidate the initial attachment of conidia. However, *C. lindemuthianum* conidia do not appear to secrete ECM onto the substratum at any stage of germination (Pain *et al.* 1996).

In many *Colletotrichum* species, the development of germ-tubes and melanised appressoria is associated with secretion of a fibrillar ECM, which forms a thick sheath around these infection structures (Jones *et al.* 1995, Kozar & Netolitzky 1978, van Dyke & Mims 1991). Both germ-tubes and appressoria adhere more strongly than conidia, and the ECM they produce remains firmly bound to the substratum after mechanical removal of the cells, suggesting it may function as an adhesive (Pain *et al.* 1996). In previous studies, we have examined the ultrastructure and composition of

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the ECM around the germ-tubes and appressoria of the bean anthracnose pathogen, *C. lindemuthianum*. Long, hair-like fimbriae, similar to those observed in *Microbotryum violaceum* and other fungi (Celerin & Day 1998), project into the ECM from the surface of germ-tubes and appressoria (Pain *et al.* 1996). In addition, cytochemistry with anionic colloidal gold, lectins and monoclonal antibodies (MAbs) has shown that the germ-tube/appressorium ECM contains basic proteins,  $\alpha$ -linked galactose residues and two large glycoproteins ( $M_r$  133 000 and 146 000) that are recognised by MAb UB26 (Pain *et al.* 1996).

The present paper describes the MAb, UB31, which was raised to infection structures of *C. lindemuthianum* isolated from bean tissues and shown to bind to germ-tubes and appressoria, but not conidia or intracellular infection hyphae (Hutchison *et al.* 1996). In a preliminary study, immunofluorescence showed that UB31 labelled the ECM surrounding appressoria (O'Connell *et al.* 1996), and the antibody was subsequently used to isolate appressoria from infected tissue by immunomagnetic separation (Hutchison *et al.* 2000). Here, we show that this antibody recognises a high  $M_r$  glycoprotein that may be linked to melanin. Comparative localisation studies indicated that the glycoproteins recognised by UB26 and UB31 occupy different regions of the ECM around germ-tubes and appressoria, demonstrating that these materials are heterogeneous in composition. In addition, evidence is presented that the glycoprotein recognised by UB31 may contribute to germling adhesion.

## MATERIALS AND METHODS

### Fungal and plant material

The *Colletotrichum* species used in this study and their sources are listed in Table 1. All cultures were maintained as described by Pain *et al.* (1992), except that *C. destructivum* and *C. sublineolum* were cultured at 25 °C and the latter was grown on quarter-strength potato dextrose agar. For production of germlings *in vitro*, conidial suspensions ( $5 \times 10^5$  spores ml<sup>-1</sup>) were prepared in distilled water and placed on glass slides,

glass plates (22 × 22 cm) or polycarbonate membranes (Pain, O'Connell & Green 1995, Pain *et al.* 1996). Conidia were allowed to germinate for 20 h at 17 °, except for *C. destructivum* and *C. sublineolum*, which were incubated at 25 °. For liquid cultures, conidia of *C. lindemuthianum* race  $\gamma$  were suspended in 0.1% (w/v) glucose in distilled water to a final concentration of approximately  $1 \times 10^4$  spores ml<sup>-1</sup>. Flasks containing 100 ml of spore suspension were incubated at 20 ° on a rotary shaker for 4–5 d and the mycelia harvested by filtration. For production of infected tissues, excised leaves of *Phaseolus vulgaris* cv. 'La Victoire' were brush-inoculated with a spore suspension of *C. lindemuthianum* race  $\gamma$  (Pain *et al.* 1994a). After 4 d, some leaves were homogenised and fungal structures isolated by isopycnic centrifugation (IPC; Pain *et al.* 1994a).

### Monoclonal antibodies

The MAb UB31 was raised in mice against *Colletotrichum lindemuthianum* infection structures isolated from infected bean leaves and is an IgG1 antibody with kappa light chains (Hutchison *et al.* 1996). Other MAbs used were UB26, which recognises a protein epitope on two glycoproteins restricted to the ECMs around germ-tubes and appressoria of *C. lindemuthianum* (Pain *et al.* 1996) and UBIM22, recognising rat bone cells (Hughes *et al.* 1999), which was used as a negative control.

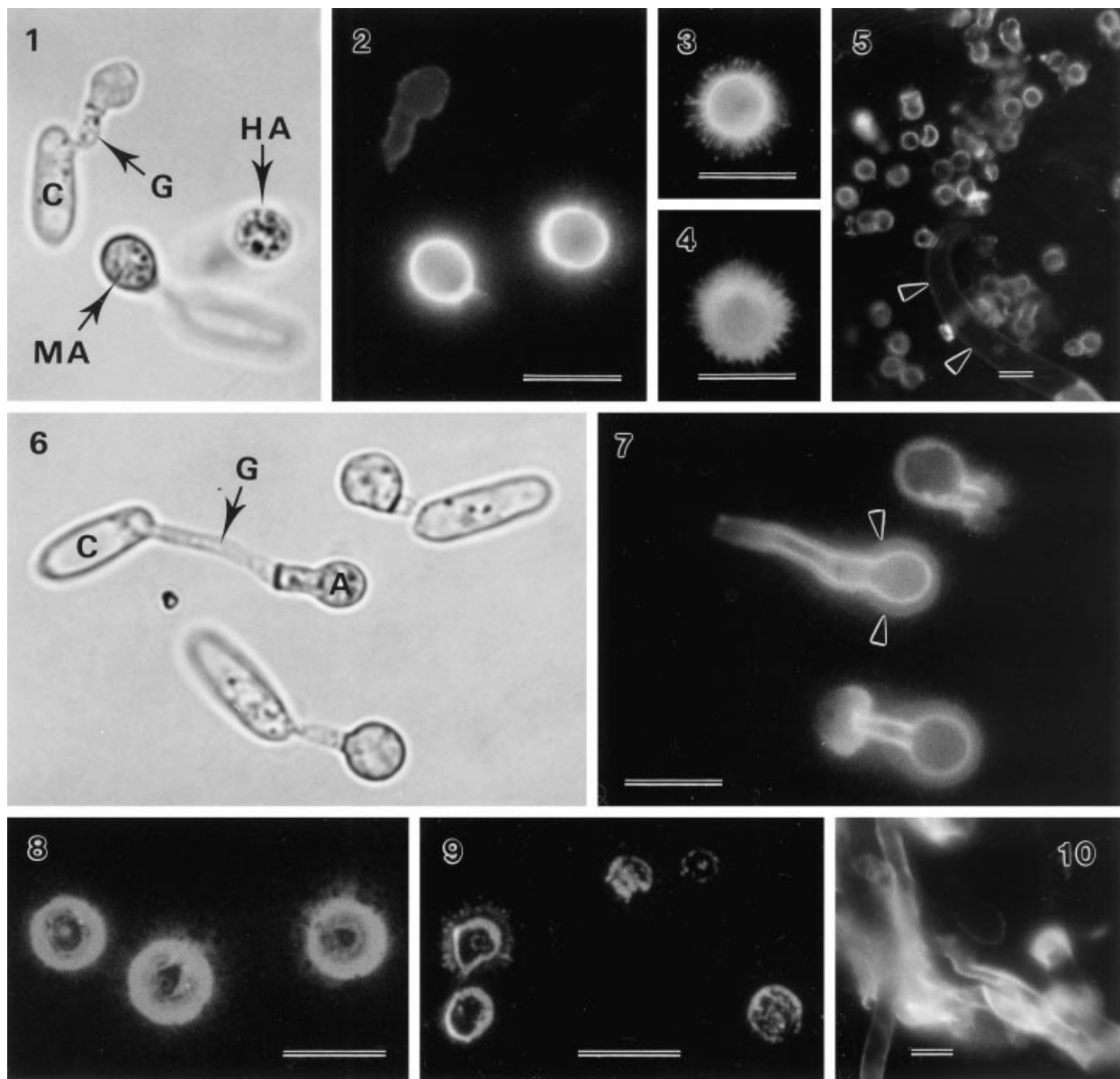
### Immunofluorescence labelling

MAb binding to cells was detected by indirect immunofluorescence microscopy (IIF) using goat anti-mouse IgG antibodies conjugated with fluorescein isothiocyanate. Germlings on glass slides were either labelled intact or after ultrasonic disruption (Pain *et al.* 1996). Infection structures were labelled *in situ* within permeabilised epidermal strips taken from infected bean leaves (Perfect *et al.* 1998). Samples of mycelium from liquid cultures were placed in the wells of Nunc Maxisorp microtitre plates (Gibco BRL, Paisley) for IIF labelling and teased apart using fine forceps before observation. Labelled cells and tissues were mounted in Vectashield

**Table 1.** Origins of the *Colletotrichum* species used.

Species	LARS Collection no.	Original Collection no.	Isolated from	Country of origin
<i>destructivum</i> <sup>a</sup>	709	CBS 520.97	<i>Medicago sativa</i>	Saudi Arabia
<i>gloeosporioides</i> <sup>b</sup>	224	IMI 337955	<i>Vigna unguiculata</i>	Nigeria
<i>lindemuthianum</i> <sup>c</sup>	129	ATCC 56987	<i>Phaseolus vulgaris</i>	Europe
<i>magna</i> <sup>d</sup>	688	CBS 519.97	<i>Citrullus lanatus</i>	USA
<i>malvarum</i> <sup>e</sup>	629	CBS 518.97	<i>Sida spinosa</i>	USA
<i>orbiculare</i> <sup>f</sup>	414	104-T	<i>Cucumis sativa</i>	Japan
<i>orbiculare</i> <sup>g</sup>	710	79215	<i>Cucumis sativa</i>	Japan
<i>sublineolum</i> <sup>h</sup>	896	NRI 74	<i>Sorghum bicolor</i>	Zambia

Cultures supplied by: <sup>a</sup>A. A. Al-Rokaibah, College of Agriculture, Buraydah, Quassim, Saudi Arabia; <sup>b</sup>K. Carwell, International Institute of Tropical Agriculture, Ibadan, Nigeria; <sup>c</sup>Race gamma, Research Institute for Plant Protection, Wageningen, The Netherlands; <sup>d</sup>R. J. Rodriguez, Western Fisheries Research Center, USGS/BRD, USA; <sup>e</sup>D. O. TeBeest, Department of Plant Pathology, University of Arkansas, Fayetteville, USA; <sup>f</sup>Y. Kubo, Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Japan; <sup>g</sup>A. Julian, Natural Resources Institute, Chatham, UK.



**Figs 1–10.** Immunofluorescence labelling of infection structures and mycelia of *Colletotrichum lindemuthianum* with MABs UB31 and UB26, viewed with bright-field (Figs 1 and 6) or epi-fluorescence microscopy. Bars = 10  $\mu$ m. **Figs 1–2.** Conidia germinating on a glass slide. UB31 strongly labels a narrow ring of ECM around an immature, hyaline appressorium (HA) and a mature, melanised appressorium (MA). Germ-tubes (G) are only weakly labelled and conidia (C) are not labelled. **Figs 3–4.** Appressoria formed on glass slides. UB31 labels fibrillar structures radiating from the cell surface. **Fig. 5.** Appressoria on the surface of a bean leaf are strongly labelled by UB31. Arrow heads indicate an autofluorescent leaf trichome. **Figs 6–7.** Conidia germinating on a glass slide. UB26 labels both germ-tubes (G) and appressoria (A) and a broad halo of ECM surrounding them (arrow heads), but conidia (C) are not labelled. **Figs 8–9.** Rings of ECM marking the previous positions of appressoria after complete removal of germlings by ultrasonication. UB26 (Fig. 8) labels broader areas of ECM than UB31 (Fig. 9). **Fig. 10.** UB31 labels mycelium growing in liquid culture (0.1% glucose).

antifade solution (Vector Laboratories, Peterborough) and examined with bright-field, differential interference contrast (DIC) or epi-fluorescence microscopy (Pain *et al.* 1994a).

#### TEM and immunogold labelling

Germlings growing on polycarbonate membranes were cryo-fixed by plunging into liquid propane, freeze-substituted in pure acetone, without any chemical fixatives, and embedded in LR White resin at  $-20^{\circ}$

(Pain *et al.* 1995). For immunogold labelling, ultrathin sections were collected on Formvar-coated gold slot grids and treated with MABs followed by goat anti-mouse IgG antibodies conjugated with 10 nm colloidal gold (Pain *et al.* 1994b). Sections were then stained with uranyl acetate and lead citrate and viewed with an Hitachi H7000 TEM.

#### SDS-PAGE and Western blotting

Protein samples were extracted from germlings scraped

off glass plates, infection structures isolated from bean leaves by IPC or homogenates of mycelium scraped from the surface of 7 d-old cultures grown on Mathur's agar medium (Mathur, Barnett & Lilly 1950, Pain *et al.* 1992). Proteins were then analysed by SDS-PAGE and Western blotting as described by Pain *et al.* (1995), except that the samples were diluted in non-reducing sample buffer, i.e. without  $\beta$ -mercaptoethanol.

### Antigen modification

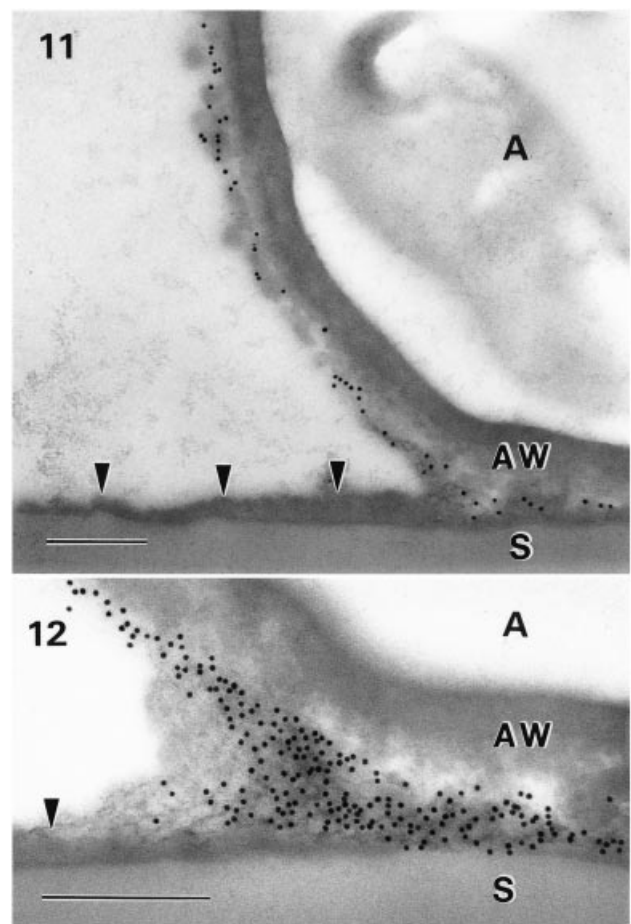
The effects of periodate oxidation and protease digestion on antigens were assessed using ELISA. Microtitre plates (Nunc, Maxisorp) were coated with infection structures isolated from bean leaves by IPC or mycelial homogenates as described by Pain *et al.* (1992). For periodate oxidation, wells were treated with 100  $\mu$ l of 20 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5) for 18 h at 4 °. Control wells received acetate buffer only. After washing four times with acetate buffer and once with phosphate-buffered saline (PBS, i.e. 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4), plates were fixed with 0.5% (v/v) glutaraldehyde and developed as described by Pain *et al.* (1992). For protease digestion, wells were treated with 100  $\mu$ l (equivalent to 12 units of activity) of Protease XIV (Sigma Chemical) in PBS for 1 h at 37 °. Control wells received PBS only. Following digestion, the enzyme was removed by rinsing the wells four times in PBS before fixation and development as above. The effect of deglycosylating antigens was assessed by Western blotting. Proteins from mycelia were treated with either peptide-N-glycosidase (PNGase) or endo-glycosidase H (endo-H; Oxford Glycosystems) prior to separation by SDS-PAGE and Western blotting (Pain *et al.* 1996).

## RESULTS

### Distribution of UB31 antigens in *C. lindemuthianum*

IIF showed that UB31 strongly labelled a narrow ring of ECM around the base of appressoria formed *in vitro*, but germ-tubes were only weakly labelled and conidia were not labelled (Figs 1–2). In some cases, fibrillar structures radiating out from the surface of appressoria were labelled (Figs 3–4). The antigen recognised by UB31 was present at all stages of appressorial differentiation, as indicated by labelling of both immature, hyaline appressoria and mature, melanised appressoria (Figs 1–2). IIF of epidermal strips taken from bean leaves infected with *C. lindemuthianum* showed that UB31 strongly labelled appressoria but not conidia or intracellular hyphae (Fig. 5). Labelling by UB31 was specific because the control antibody UBIM22 did not label any fungal structures, as shown previously (Pain *et al.* 1992).

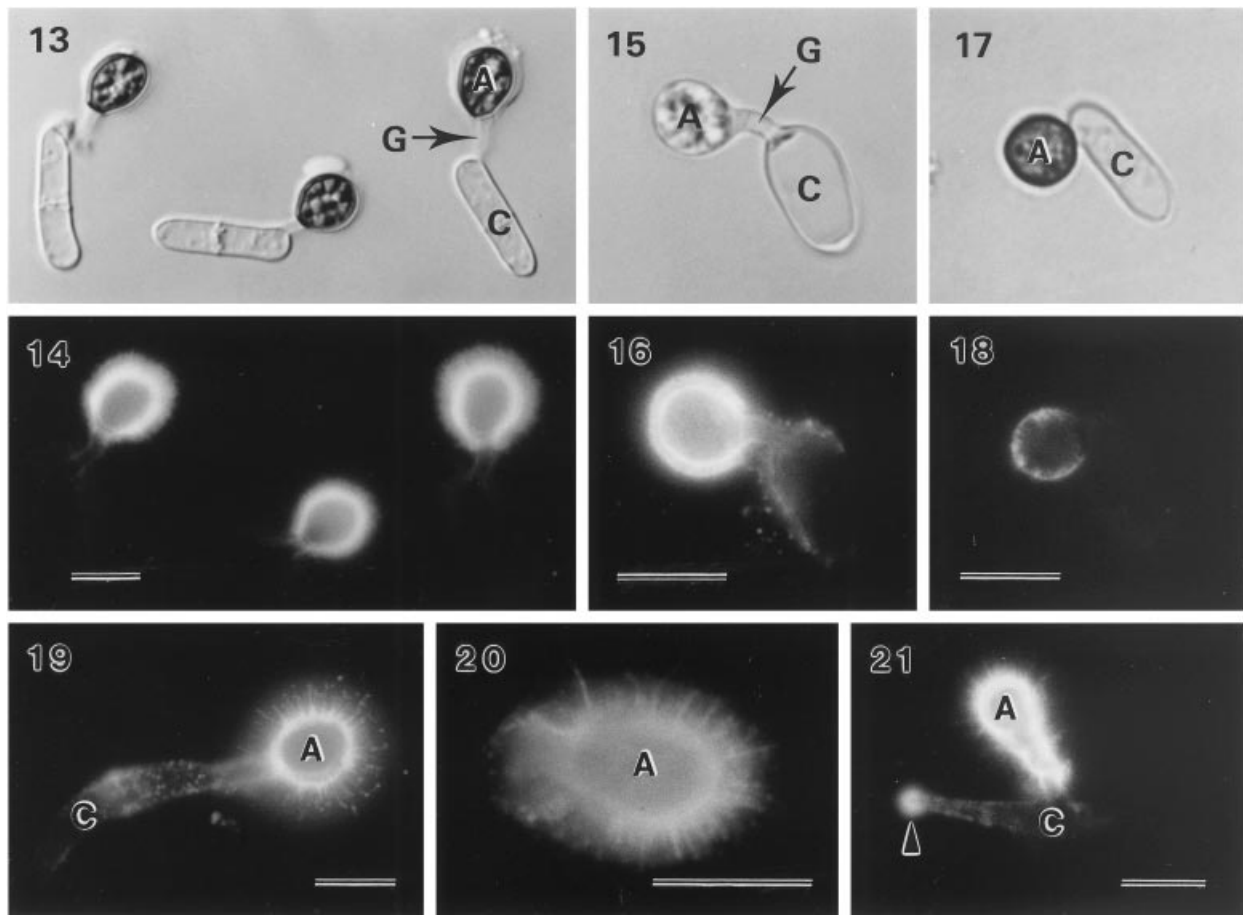
The pattern of labelling obtained with MA b UB26 was similar to that reported previously (Pain *et al.* 1996). Unlike UB31, this antibody labelled germ-tubes



**Figs 11–12.** TEM-immunogold labelling with MA b UB31 of appressoria (A) of *Colletotrichum lindemuthianum* formed on polycarbonate membranes. Bars = 0.2  $\mu$ m. UB31 labels a thin layer of ECM coating the cell surface and accumulating at the contact interface with the substratum (S). Electron-opaque ECM extending outwards over the substratum (arrow heads) and the appressorial cell wall (AW) are not labelled.

and appressoria equally strongly and revealed a much broader halo of ECM around germ-tubes and appressoria (Figs 6–7). As with UB31, conidia were not labelled by UB26. Following removal of germlings from glass slides by ultrasonication, UB26 labelled large circular areas of ECM marking the previous positions of appressoria (Fig. 8), whereas UB31 labelled narrower rings of appressorial ECM (Fig. 9). Mycelia from liquid cultures were labelled strongly by UB31 (Fig. 10), whereas UB26 labelled mycelia very sparsely (results not shown).

TEM-immunogold labelling of germlings on polycarbonate membranes showed that UB31 bound to a thin layer of ECM coating the surface of appressoria (Fig. 11) and at the contact interface between the appressorium and substratum (Fig. 12). However, there was no labelling of the ECM spreading outwards from the appressorium over the substratum (Fig. 11), and neither the appressorial cell wall nor cytoplasm were labelled (Figs 11–12). Ultrastructural preservation of the cytoplasm and membrane contrast were poor in these preparations because the germlings were freeze-sub-



**Figs 13–21.** Immunofluorescence labelling with MAb UB31 of infection structures produced by three different *Colletotrichum* species on glass slides. Viewed with DIC (Figs 13 and 15), bright-field (Fig. 17) or epi-fluorescence microscopy (Figs 14, 16 and 18–21). Bars = 10  $\mu$ m. **Figs 13–14.** ECM around appressoria (A) of *C. magna* is strongly labelled. **Figs 15–16.** ECM around the hyaline appressorium (A) of an albino mutant (79215) of *C. orbiculare* is strongly labelled. **Figs 17–18.** A melanised appressorium (A) of wild-type *C. orbiculare* (strain 104-T) is only weakly labelled. **Figs 19–21.** UB31 labels fibrillar structures radiating from the surface of *C. sublineolum* appressoria (A) and small areas of ECM (arrow head) at the apex of the conidium (C).

stituted in acetone, without any chemical fixatives. This was necessary because preliminary experiments showed that UB31 antigens did not survive fixation with glutaraldehyde or osmium tetroxide.

#### **Distribution of UB31 antigens in other *Colletotrichum* species**

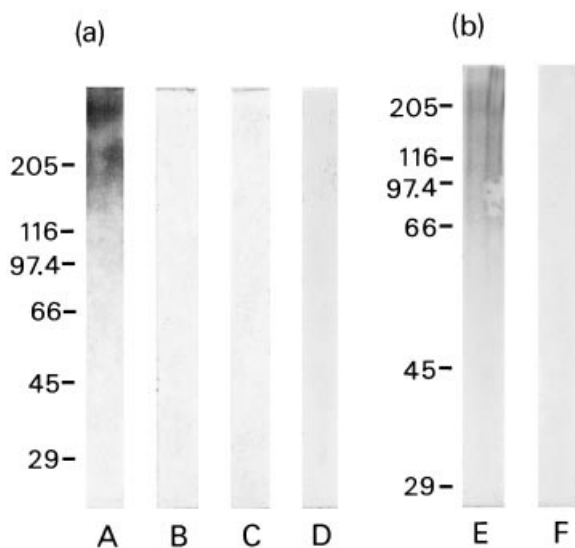
IIF was used to determine whether UB31 labelled the surfaces of germinated conidia, germ-tubes and appressoria of other *Colletotrichum* species growing on glass slides. UB31 bound to all of the *Colletotrichum* species examined, namely *C. destructivum*, *C. gloeosporioides*, *C. orbiculare*, *C. magna*, *C. malvarum*, and *C. sublineolum*. In most species, the pattern of labelling resembled that of *C. lindemuthianum* described above, i.e. the ECMs around germ-tubes and appressoria, but not conidia, were labelled (Figs 1–2). Labelling was strongest on the appressoria of *C. magna* (Figs 13–14). The hyaline appressoria of a melanin-deficient mutant of *C. orbiculare* (Figs 15–16) were labelled more strongly than melanised appressoria of the wild-type of this

species (Figs 17–18). In *C. sublineolum*, UB31 labelled fibrillar structures radiating out from germ-tubes and appressoria (Figs 19–20). Small areas of ECM released onto the substratum at one or both apices of some conidia were also labelled in this species (Fig. 21).

#### **Characterisation of the antigens recognised by UB31**

Proteins extracted from homogenates of *Colletotrichum lindemuthianum* infection structures produced *in vitro* and IPC preparations of infection structures isolated from infected bean leaves were separated by SDS-PAGE for Western blotting (Fig. 22). These samples showed no labelling after incubation with UB31. However, in Western blots of mycelial homogenates, UB31 labelled a smear of high  $M_r$  proteins (> 200000). Binding was only observed when samples were separated in non-reducing conditions. Blots probed with the control antibody UBIM22 were not labelled (Fig. 22).

In Western blots of proteins extracted from appressoria of the albino mutant of *C. orbiculare*, UB31 labelled a smear of proteins,  $M_r$  70000 to 250000 (Fig.



**Fig. 22.** (a) Western blots showing labelling by MAbs UB31 (A–C) and UBIM22 (D) of *Colletotrichum lindemuthianum* proteins extracted from mycelia grown on Mathur's agar medium (A), infection structures isolated from bean leaves by IPC (B) or infection structures grown *in vitro* (C and D). UB31 only labels proteins of  $M_r > 200\,000$  in mycelial samples (A). The control antibody UBIM22 does not label any proteins (D). (b) Western blots showing labelling by UB31 of proteins extracted from infection structures of *C. orbiculare* grown *in vitro*. A smear of proteins ( $M_r$  70 000–250 000) is labelled in samples prepared from the albino mutant strain 79215 (E), but no proteins are labelled in samples from the melanised wild-type strain 104-T (F). All proteins were separated by SDS-PAGE under non-reducing conditions.  $M_r$  ( $\times 10^3$ ) are indicated to the left of the blots.

**Table 2.** Effects of periodate oxidation and protease digestion of *Colletotrichum lindemuthianum* antigens on the binding of MAb UB31, as determined by ELISA.

Sample	Treatment	Absorbance
Infection structures	Control	$0.27 \pm 0.08$
	Periodate	$0.04 \pm 0.01$
Mycelium	Control	$0.71 \pm 0.04$
	Periodate	$0.04 \pm 0.01$
Infection structures	Control	$0.24 \pm 0.04$
	Protease	$0.05 \pm 0.02$
Mycelium	Control	$0.34 \pm 0.04$
	Protease	$0.06 \pm 0.02$

ELISA plates were coated with either infection structures isolated from leaves by IPC or mycelial homogenates and then treated with periodate or protease before sequential addition of MAb UB31, alkaline phosphatase-conjugated rabbit anti-mouse IgG, and substrate. Absorbance values ( $A_{405\text{ nm}}$ ) are expressed as means and standard deviations of three replicate determinations. The control MAb UBIM22 showed no binding to the samples.

22). In contrast, samples prepared from the pigmented appressoria of wild-type *C. orbiculare* were not labelled in Western blots (Fig. 22). UB31 failed to recognise any proteins in extracts of appressoria of *C. magna*, *C. gloeosporioides* or *C. sublineolum* (results not shown). Samples were not prepared from *C. destructivum* or *C. malvarum* due to insufficient sporulation.

The effect of periodate oxidation or protease digestion on the binding of UB31 to IPC preparations and mycelial homogenates of *C. lindemuthianum* was determined by ELISA. Binding to both samples was reduced, but not eliminated, after exposure of the antigens to periodate (Table 2). Similarly, UB31 binding was reduced but not eliminated following treatment of the antigens with protease (Table 2). These results suggest that UB31 recognises a glycoprotein. Deglycosylation of proteins from mycelial homogenates with either PNGase or endo-H had no effect on UB31 binding, suggesting that the carbohydrate side-chains are unlikely to be *N*-linked (results not shown).

## DISCUSSION

In this paper, we have used the MAb UB31 to analyse the organisation and composition of the ECMs produced by the infection structures of *Colletotrichum* species. Comparisons have been made with the labelling pattern of a second antibody, UB26, which was used in a previous study (Pain *et al.* 1996). Although UB31 and UB26 both labelled the same fungal cell types (germ-tubes, appressoria and mycelia), their labelling patterns with IIF, TEM-immunogold labelling and Western blotting were different.

In previous studies using UB26 and lectin probes, we showed that the ECMs surrounding germ-tubes and appressoria of *C. lindemuthianum* are very similar in composition but differ from those of conidia and the intracellular hyphae formed inside host cells (O'Connell *et al.* 1996, Pain *et al.* 1996). The present results with UB31 confirm these differences in composition between fungal cell types, and reveal two further features. First, there were differences in the abundance of the antigens recognised by the antibodies on germ-tubes and appressoria. Thus, antigens recognised by UB31 were much more abundant on appressoria than on germ-tubes, while those recognised by UB26 were equally abundant on both. Second, the glycoproteins recognised by these two MAbs are located in different regions of the ECM. Thus, IIF and TEM-immunogold labelling showed that the UB31 antigen was concentrated in a thin layer of ECM just outside the appressorial wall, whereas UB26 antigens are distributed throughout the ECM and fungal cell wall (Pain *et al.* 1996). These results indicate that the ECM of *C. lindemuthianum* is heterogeneous in composition, with some glycoproteins spreading further from the cell surface than others. The proteinaceous ECM around germ-tubes of *Cochliobolus* spp. is also heterogeneous, comprising two sharply defined layers (Apoga & Jansson 2000). Sugui *et al.* (1999) suggested that in *Cochliobolus heterostrophus*, multiple layers of ECM are deposited at different stages of germination. However, this does not appear to be the case in *C. lindemuthianum*, because both UB26 and UB31 antigens are produced simultaneously, as soon as germ-tubes start to emerge from conidia (S. Rawlings & J. R. Green, unpubl.).

ECM containing the glycoprotein recognised by UB31 was not removed from glass substrata after ultrasonic disruption of germlings, suggesting that this glycoprotein may contribute to the adhesion of germ-tubes and appressoria. However, it is unlikely to be the only adhesive component involved since the 'footprints' of ECM were also labelled by UB26, as reported previously (Pain *et al.* 1996). UB31 labelled fibrillar structures radiating from the surface of *C. sublineolum* and *C. lindemuthianum* appressoria that were similar in length and arrangement to the fimbriae observed by TEM in the latter species after negative staining (Pain *et al.* 1996). These hair-like surface appendages have been detected in a wide range of fungi (Celerin & Day 1998). However, although fimbriae (pili) have been shown to be essential for the adhesion of some plant pathogenic bacteria to host surfaces (Romantschuk *et al.* 1993), it remains unclear if they have any role in fungal pathogenesis.

MAB UB26 recognises a protein epitope carried on two glycoproteins ( $M_r$  133000 and 146000) present on the germ-tubes, appressoria and mycelium of *C. lindemuthianum* (Pain *et al.* 1996). In contrast, UB31 only labelled glycoproteins ( $M_r > 200000$ ) from the mycelium of this fungus in Western blots. However, ELISA experiments suggested that UB31 recognises a similar epitope present in both mycelium and infection structures, that was susceptible to treatments which affect carbohydrate and protein structure. It therefore appears that in germ-tubes and appressoria, UB31 binds to an ECM component that is not extractable with SDS-sample buffer. Glycoproteins recognised by UB31 could be extracted from the hyaline appressoria of a melanin-deficient mutant of *C. orbiculare* but not from melanised appressoria of the wild-type, suggesting that melanisation affects their extractability. In *Colletotrichum*, the melanin polymer is formed by oxidative cross-linking of 1,8-dihydroxynaphthalene (DHN; Kubo & Furusawa 1991). There is evidence that when phenolic molecules such as DHN undergo oxidative cross-linking in the presence of proteins and amino acids, the latter can become incorporated into the polymer (Leatham, King & Stahmann 1980, Martin & Haider 1980). During the melanisation of appressoria, it is therefore possible that ECM proteins, including the UB31 antigen, become chemically cross-linked into the melanin polymer, and thus insolubilised. This may contribute to the UB31 glycoprotein becoming restricted to the region adjacent to the melanised appressorial wall. Since UB26 antigens appear not to be constrained by this binding to melanin, they may diffuse further away from the appressorial wall. The cross-linking of proteins to melanin is also likely to reduce their accessibility to antibodies, which could explain why appressoria of the albino mutant were labelled more strongly by UB31 than those of the wild-type.

To further study the effect of melanisation on the extractability of glycoproteins recognised by UB31, we

used the melanin biosynthesis inhibitor, tricyclazole (Kubo & Fusarawa 1991). *C. lindemuthianum* germlings were incubated in 100  $\mu$ M tricyclazole, after which proteins were extracted and tested for UB31 binding in Western blots. Although the appressoria were not melanised after this treatment, no glycoproteins could be detected with UB31 (results not shown). The albino mutant used in this study (79215) is blocked early in the pathway of melanin synthesis, probably at, or before, pentaketide cyclization (Kubo & Fusarawa 1991). In contrast, tricyclazole inhibits the later conversion of 1,3,8-trihydroxynaphthalene to vermeline, causing the accumulation of a shunt product, 3,4-dihydro-4,8-dihydroxy-1(2H)naphthalenone (Kubo & Fusarawa 1991). It is possible that the glycoproteins recognised by UB31 become bound to this shunt product, which may affect their extractability with SDS. However, this hypothesis needs further investigation.

Within the genus *Colletotrichum*, UB26 shows restricted binding to *C. lindemuthianum*, *C. malvarum*, *C. orbiculare* and *C. trifolii* (Pain *et al.* 1996), which are thought to be *formae speciales* of a single aggregate species (Pain *et al.* 1992). In contrast, UB31 bound to the germ-tubes and appressoria of all *Colletotrichum* species tested, suggesting there are biochemical similarities between the ECMs of these fungi. UB31 could therefore be a valuable probe for the specific detection of appressoria in a wide range of anthracnose diseases, e.g. by immunofluorescence labelling of appressoria on plant surfaces (see Fig. 5). UB31 can also be used for the affinity purification of appressoria by immunomagnetic separation, as described elsewhere (Hutchison *et al.* 2000).

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