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Localization of chitinolytic activities in *Fagus sylvatica* mycorrhizas

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Abstract Localization of chitinolytic activities in *Fagus sylvatica* (beech) mycorrhizas was examined using a range of fluorogenic 4-methylumbelliferyl [4-MU-$(GlcNAc)_{1–4}$] substrates in order to distinguish between exochitinase, endochitinase and β-*N*–acetylglucosaminidase activities. The validity of the technique was confirmed using onion epidermis cells. In the beech mycorrhiza, endochitinase activity was not detectable above background fluorescence. Exochitinase activity was detected in the fungal sheath and the Hartig net. β-*N*–Acetylglucosaminidase activity was also mainly associated with the fungal sheath and Hartig net. Individual fungal hyphae extending from these structures also showed substantial β-*N*–acetylglucosaminidase activity. The cortical cell walls of the host in the Hartig net region also fluoresced brightly. The localization of β-*N*–acetylglucosaminidase activity was confirmed using a chromogenic histochemical reagent, 5-bromo-4-chloro-3-indolyl-*N*–acetyl-β-D-glucosaminide (X-GlcNAc).

Key words Ectomycorrhizae · Chitinolytic activities · Localization · 4-Methylumbelliferyl substrates [4-MU-$(GlcNAc)_{1–4}$] · 5-Bromo-4-chloro-3-indolyl-*N*–acetyl-β-D-glucosaminide (X-GlcNAc)

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Introduction

Chitin occurs as a major component in the cell walls of fungi belonging to the Basidiomycotina, Ascomycotina, Deuteromycotina and Zygomycotina (Wessels and Sietsma 1981; Gooday 1990a). Enzymatic hydrolysis of chitin to its monomer β-*N*–acetylglucosamine occurs by a binary chitinolytic enzyme system. Chitinases (EC 3.2.1.14) hydrolyse the chitin polymer to oligosaccharides. Endochitinase activity involves random cleavage at internal points in the chitin chain while exochitinase activity is progressive at non-reducing ends with release of diacetylchitobiose units (Robbins et al. 1988). β-*N*–Acetylglucosaminidase (EC 3.2.1.30) hydrolyses *N*,*N*′-diacetylchitobiose to release *N*–acetylglucosamine. All chitin-containing fungi investigated thus far produce chitinases (Gooday 1990b). A number of roles for fungal chitinase activity have been suggested, including regulation of the formation of crystalline chitin in its hydrogen-bonded form, apical growth, spore germination and nutrition (Gooday 1990b,c). We have previously shown that several ectomycorrhizal basidiomycetes can secrete chitinolytic activities when grown in liquid culture, although there was considerable variation in activities recorded between isolates (Hodge et al. 1995a). Other fungal chitinolytic systems that have been investigated include those from the ericoid endophyte *Hymenoscyphus ericae* (Mitchell et al. 1992), the insect pathogen *Metarhizium anisopilae* (St Leger et al. 1986) and the mycoparasite *Trichoderma harzianum* (Ulhoa and Peberdy 1991, 1992).

Higher plants contain chitinase despite the absence of its substrate, chitin. Plant chitinase activity is induced in response to a wide range of abiotic or physical stresses (Collinge et al. 1993; Graham and Sticklen 1994). However, it is the increased chitinase activity in response to pathogen invasion which has received most attention (Boller 1985; Collinge et al. 1993), supported by the demonstration of antifungal activity in vitro of certain classes of plant chitinase (Schlumbaum et al.

1986; Roberts and Selitrennikoff 1988; Sela-Buurlage et al. 1993). Plants also contain constitutive amounts of β-*N*–acetylglucosaminidase activity which may be induced in response to chitinous pathogens (Hodge et al. 1995b). As root systems of higher plants are also infected by mycorrhizal fungi, this has stimulated research into the role of chitinase during both arbuscular mycorrhizal (AM) (Spanu et al. 1989; Dumas-Gaudot et al. 1992, 1994; Vierheilig et al. 1993, 1994) and ectomycorrhizal (Albrecht et al. 1994a,b,c; Hodge et al. 1995b) formation. However, comparatively little is known about the localization of chitinolytic activities in intact ectomycorrhizas.

The aim of this present study was to test a range of fluorogenic 4-methylumbelliferyl substrates (McCreath and Gooday 1992) for their ability to localize chitinolytic activities using fully formed ectomycorrhizal material. In addition, a newly available chromogenic histochemical stain, 5-bromo-4-chloro-3-indolyl-*N*–acetyl-β-D-glucosaminide (X-GlcNAc), which is a substrate for β-*N*–acetylglucosaminidase activity, was used to confirm the localization of β-*N*–acetylglucosaminidase activity detected by the fluorogenic substrate 4-MU-GlcNAc. As the 4-methylumbelliferyl substrates have not previously been used for microscopical study of plant sections, their potential was tested on onion epidermis, commonly used as a model plant tissue due to the ease of preparation of a monolayer of uniform cells. In the present study, onion epidermis was found to have no autofluorescence at the excitation and emission wavelengths used.

Materials and methods

Mycorrhizas of beech (*Fagus sylvatica* L.) were collected from the surface of organic horizons of a 150-year-old beech stand near Aberdeen. We chose to use field material in this preliminary investigation in order to obtain established ectomycorrhizas free from the possible artefacts of in vitro synthesis. Beech mycorrhizas are readily identified and easily sectioned. A uniform type (*Lactarius* spp., see Agerer 1987) was selected. The usefulness of the 4-methylumbelliferyl substrates to localize chitinolytic activities was tested using onion epidermis cells. Preparations were examined with a Zeiss Axiophot Photomicroscope, using differential interference contrast and/or fluorescence microscopy. For fluorescence microscopy under UV light, a Zeiss filter set of excitation 365 nm, beam splitter 395 nm and barrier filter 420 nm was used.

Preparation of substrates for chitinolytic activities

The fluorogenic substrates were 4-methylumbelliferyl glycosides of *N*–acetylglucosamine oligosaccharides [4-MU-GlcNAc, 4-MU-$(GlcNAc)_2$, 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_4$] (McCreath and Gooday 1992) supplied by Sigma Chemical Co. (monomer, dimer and trimer) and Janssen Biochimica (tetramer). The use of these substrates allows discrimination between β-*N*–acetylglucosaminidase (hydrolysing 4-MU-GlcNAc), exochitinase [hydrolysing 4-MU-$(GlcNAc)_2$] and endochitinase [hydrolysing 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_4$] (Robbins et al. 1988; McCreath and Gooday 1992). Stock solutions of monomer, dimer and trimer were prepared at a concentration of 0.8 mM in distilled water. Tetramer (0.8 mM) was prepared in 50% ethanol. Stock solutions were stored at –20 °C.

The chromogenic histochemical reagent, 5-bromo-4-chloro-3-indolyl-*N*–acetyl-β-D-glucosaminide (X-GlcNAc), which is a substrate for β-*N*–acetylglucosaminidase activity, was prepared at a concentration of 0.8 mM in 50% ethanol. Stocks were stored at –20 °C

Preparation of onion tissue incubated with 4-MU-GlcNAc

The inner epidermal layer from an onion was carefully peeled, cut into approximately 7 × 2-mm pieces and mounted on a glass slide in a solution containing 30 µl sorbitol (0.55 M) (to prevent excess cell turgor which causes the preparation to curl), 12.5 µl MES buffer (0.1 M, pH 5.0) and 12.5 µl 4-MU-GlcNAc (0.8 mM). The slide was supported on glass blocks over damp filter paper inside a Petri dish and incubated for 30 min at room temperature. A coverslip was then placed on top of the preparation and the edges sealed with a warm lanolin/vaseline/paraffin wax (1/1/1) mixture. For the controls, 12.5 µl of 4-methylumbelliferone (0.8 mM) was added instead of 4-MU-GlcNAc.

Beech mycorrhiza material

For longitudinal sectioning, beech mycorrhizas were mounted in 5% sodium alginate and gelled by immersion in 20 mM $CaCl_2$ for 5 min. Sections (ca. 150 µm) were cut with razor blades and mounted in 12.5 µl MES buffer (0.1 M, pH 5.0) and 12.5 µl 4-MU-$(GlcNAc)_{1–4}$ (0.8 mM) as before. Controls were treated as above. Sections were also mounted in 12.5 µl MES buffer (0.1 M,

Figs. 1–4 Photomicrographs of onion epidermis cells; *bars* 30 µm ▶ (*C* cytoplasm, *D* damaged cells, *N* nuclei)

Fig. 1 Section incubated with 4-MU-GlcNAc for 30 min and viewed by incident fluorescence optics showing β-*N*–acetylglucosaminidase activity associated mainly with the cytoplasm (*broad arrows*) and the nuclei (*arrows*). The area of the section damaged during preparation (as indicated by the lack of cytoplasmic streaming) did not fluorescence

Fig. 2 As in Figure 1 but viewed by differential interference contrast (DIC) optics

Fig. 3 Detail from Fig. 1 clearly demonstrating β-*N*–acetylglucosaminidase activity localized in the cytoplasm (*broad arrow*)

Fig. 4 As in Fig. 3 but viewed by DIC optics

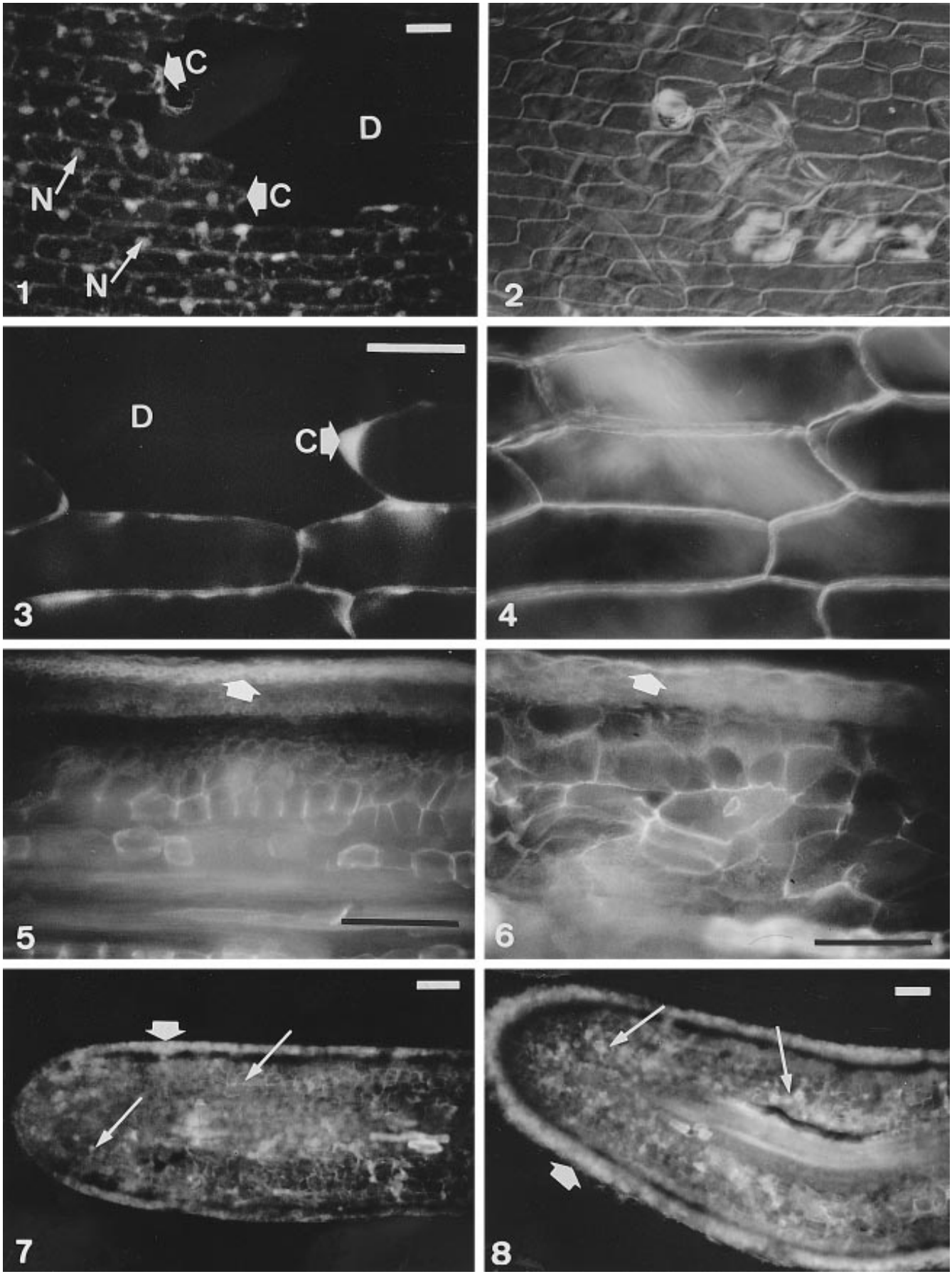
Figs. 5–8 Photomicrographs of longitudinal sections of beech mycorrhiza incubated with the fluorogenic substrate 4-MU-$(GlcNAc)_2$ or 4-methylumbelliferone (control), viewed by incident fluorescence optics; *bars* 30 µm

Fig. 5 Control section incubated for 25 min with 4-methylumbelliferone, showing autofluorescence in cortical cell walls and at the edge of the fungal sheath (*broad arrow*)

Fig. 6 Section incubated for 40 min with 4-MU-$(GlcNAc)_2$. The sheath shows exochitinase activity (*broad arrow*). The walls of cortical cells also fluoresce showing exochitinase activity

Fig. 7 Section incubated for 15 min with 4-MU-$(GlcNAc)_2$. Areas of the sheath fluoresced, indicating exochitinase activity (*broad arrow*). Note bright fluorescence within the cortex (*arrows*)

Fig. 8 Section incubated for 23 min with 4-MU-$(GlcNAc)_2$. Bright spots of exochitinase activity within the cortex are evident (*arrows*). The fungal sheath also shows exochitinase activity (*broad arrow*)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pH 5.0) and 12.5 μl X-GlcNAc (0.8 mM). This reagent is soluble and colourless until the enzymatic action of β–*N*–acetylglucosaminidase results in the release of the aglycone, which is converted rapidly to an insoluble indigo compound.

The incubation was for 90 min, 4 h or 24 h. Control slides, prior to addition of X-GlcNAc, were held over a Bunsen flame until the fluid boiled gently. This procedure was repeated three times at 1-min intervals, taking care that the preparation did not dry. The slide was left for 1 min before addition of X-GlcNAc as before.

Results

Onion epidermis cells incubated with 4-MU-GlcNAc

Fluorescence developed in the cytoplasm (Figs. 1, 3), and strongly in the region of the nuclei (Fig. 1) after 30 min incubation, but there was no fluorescence in the vacuole. Areas damaged during preparation (Figs. 2, 4), and those in which cytoplasmic streaming was not observed, did not fluoresce (Figs. 1, 3). The controls also did not fluoresce.

Beech mycorrhiza sections incubated with 4-MU-$(GlcNAc)_{1–4}$

Control sections incubated with 4-methylumbelliferone showed autofluorescence in cortical cell walls and in the outer sheath (Fig. 5). This fluorescence was distinguishable in intensity and colour from that produced after hydrolysis of the 4-MU substrates. Fluorescence of the sections incubated with 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_4$, the 4-MU substrates for endochitinase activity, was not distinguishable from that of the controls. The sections incubated with 4-MU-GlcNAc and 4-MU-$(GlcNAc)_2$, however, showed brighter areas of fluorescence than in the control sections, indicating the presence of β-*N*–acetylglucosaminidase and exochitinase activity, respectively.

The sections incubated with 4-MU-$(GlcNAc)_2$ fluoresced brightly across the entire fungal sheath (Figs. 6–8), indicating exochitinase activity. The sections incubated with 4-MU-GlcNAc also showed the strongest fluorescence in the fungal sheath (Figs. 9, 10) and in the area of the Hartig net (Fig. 11), indicating β-*N*–acetylglucosaminidase activity in these regions. Large diameter hyphae (6 μm) in the cortex (Fig. 12), probably belonging to an ascomycete (Brand 1992), fluoresced brightly (Fig. 11), as did hyphae protruding from the fungal sheath (Fig. 9).

Beech mycorrhiza sections incubated with X-GlcNAc

After a 90-min incubation, the fungal sheath stained a distinctive blue colour (Fig. 14); there was no staining of the boiled control (Fig. 13). After 4 h and 24 h, the location of the stain had not altered but the colour had intensified. β-*N*–Acetylglucosaminidase activity localized by this technique was primarily in the fungal sheath (Figs. 14–16). The large ascomycetous hyphae within the cortical cells were also stained by this method (Fig. 16).

Discussion

The 4-methylumbelliferyl substrates have proven to be highly sensitive for the detection of chitinolytic activities in biochemical assay systems (McCreath and Gooday 1992; Hodge et al. 1995a,b). In this present study, the potential of these substrates to localize chitinolytic activities in mycorrhizas was examined, after confirmation of the technique using onion epidermal cells.

β-*N*–Acetylglucosaminidase activity was localized in the cytoplasm of the onion epidermal cells in common with other plant glycosidases (Pierrot and van Wielink 1977). β-*N*–Acetylglucosaminidase was also localized in the nuclear region. These results indicate an intracellu-

Figs. 9–12 Photomicrographs of longitudinal sections of beech mycorrhiza incubated with the fluorogenic substrate 4-MU-GlcNAc and viewed by incident fluorescence optics unless otherwise stated; *bars* 30 μm

Fig. 9 Section incubated for 25 min with 4-MU-GlcNAc. A band of β-*N*–acetylglucosaminidase activity was localized in the fungal sheath (*arrowheads*). A fungal hypha protruding from the sheath also fluoresced (*arrow*)

Fig. 10 Section incubated for 25 min with 4-MU-GlcNAc. A band of β-*N*–acetylglucosaminidase activity was localized in the fungal sheath (*arrowheads*)

Fig. 11 Section incubated for 40 min with 4-MU-GlcNAc, showing β-*N*–acetylglucosaminidase activity associated with the cortical cell walls (*arrow*) and the Hartig net (*Hn*). The large fungal hypha, probably ascomycetous, protruding from the Hartig net shows considerable β-*N*–acetylglucosaminidase activity particularly at the cross walls (*arrowheads*)

Fig. 12 As in Fig. 11 but viewed by DIC

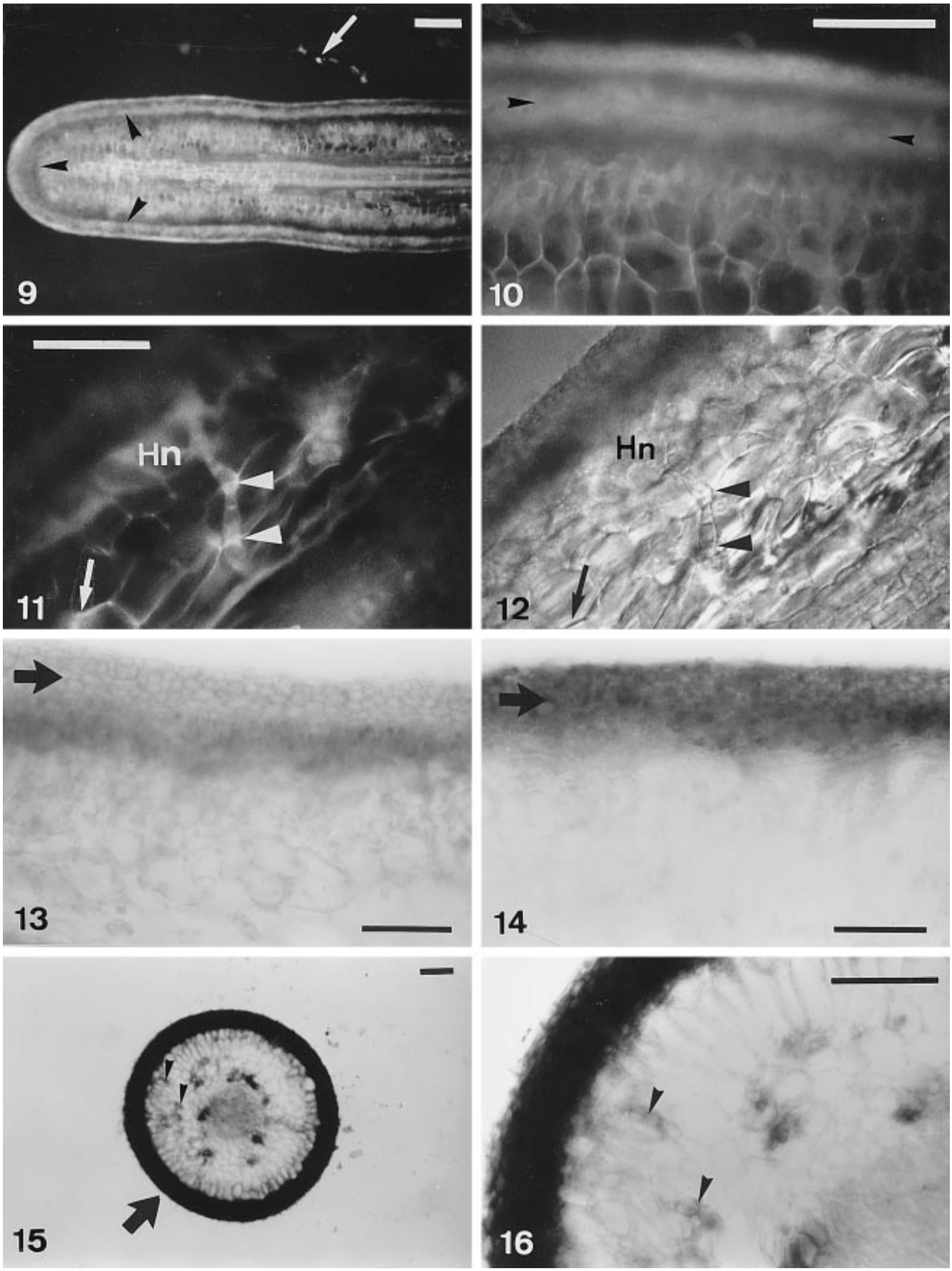
Figs. 13–16 Photomicrographs of beech mycorrhiza incubated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-*N*–acetyl-β-D-glucosaminide (X-GlcNAc) and viewed by brightfield optics; *bars* 30 μm

Fig. 13 Boiled control longitudinal section incubated for 4 h with X-GlcNAc showing no β-*N*–acetylglucosaminidase activity. The *broad arrow* indicates the fungal sheath

Fig. 14 Longitudinal section incubated for 4 h with X-GlcNAc showing β-*N*–acetylglucosaminidase activity associated with the fungal sheath (*broad arrow*), which has clearly stained compared with the control (cf. Fig. 13)

Fig. 15 Transverse section incubated with X-GlcNAc for 24 h showing β-*N*–acetylglucosaminidase activity mainly associated with the fungal sheath (*broad arrow*). Small areas of stain within the cortex are also shown (*arrowheads*)

Fig. 16 Detail from Fig. 15. The small areas of stain within the cortex are shown to be associated with large, probably ascomycete, hyphae (*arrowheads*). Faint staining of the cortical cell walls can be seen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lar compartmentalization rather than the intercellular location reported for induced acidic chitinase activity (Boller and Métraux 1988). The intracellular localization of the observed activity may be a consequence of the acidic buffer used. This low pH was chosen for this study as host-produced acidic chitinases have been shown to be important in both ectomycorrhizal (Albrecht et al. 1994a) and AM (Dumas-Gaudot et al. 1994) establishment. There are no other reports in the literature of plant *β-N*–acetylglucosaminidase or chitinase activities localized in the nuclear region. Manson et al. (1992) observed fluorescence around the nuclei of red blood cells from turbot (*Scophthalmus maximus*) using 4-MU-GlcNAc and 4-MU-$(GlcNAc)_2$ as substrates.

The exochitinase and *β-N*–acetylglucosaminidase activities localized in the beech mycorrhizas were largely associated with the fungal partner. We have previously demonstrated that these are the predominant chitinolytic activities secreted extracellularly by ectomycorrhizal fungi (Hodge et al. 1995a). The results from the present study suggest both *β-N*–acetylglucosaminidase and exochitinase are present when the fungi are in symbiosis with their host. The fluorescence observed in the cytoplasm and at the cortical cell walls was in agreement with the results from the onion epidermal sections. However, due to the problem of autofluorescence occurring at the same wavelength as that required to detect 4-MU release, and because of the highly vacuolar nature of the beech cortical cells, it was often difficult to distinguish this activity from background.

The results using the chromogenic histochemical reagent X-GlcNAc were similar to those using the 4-MU-GlcNAc substrate but the staining of the sheath was more intense. Similarly the hyphae from the secondary ascomycetous infection, and to some extent the cytoplasm and walls of some cortical cells, stained with the X-GlcNAc substrate, indicating the presence of *β-N*–acetylglucosaminidase activity. Formation of the Hartig net, which is in close contact with host cortical cells, is a dynamic process (Harley 1984), and it has been postulated that host chitinase activity is involved in Hartig net development (Sauter and Hager 1989). Although endochitinase activity was not detected in the present study, we have recently shown that *β-N*–acetylglucosaminidase activity may be an early response by the host to a chitinous pathogen challenge (Hodge et al. 1995b). It may be that the observed *β-N*–acetylglucosaminidase activity was a host response to hyphae penetrating between the cortical cells, but further investigations are required as the activity may equally well be of fungal origin.

Spanu et al. (1989) studied localization of chitinase during the early stages of the AM association between *Allium porrum* and *Glomus versiforme* using an immunocytochemical procedure. In contrast to our study, chitinase was localized in the vacuoles and extracellular spaces of both mycorrhizal and nonmycorrhizal roots. Host chitinase in the vacuole was separated from the fungus by membrane systems. In a recent study by Sela-Buurlage et al. (1993), vacuolar chitinases of tobacco plants exhibited the most effective antifungal activity. The extracellular host chitinase observed in the study by Spanu et al. (1989) was never seen to bind to the fungal cell wall, and the authors concluded that host chitinase was not important in the establishment of the symbiosis. Similarly the results of this present study suggest endochitinase activity, the predominant host chitinase activity (Boller 1988), is not important in established mycorrhizal material, as it was not detectable in our system. The activities localized in this study were predominantly of fungal origin. The ability of mycorrhizal fungi to produce such activities when in symbiosis may be important for the nutrition of the host (Leake and Read 1990).

In conclusion, we have demonstrated *β-N*–acetylglucosaminidase and exochitinase activity in established mycorrhizal material by use of 4-MU-$(GlcNAc)_{1–4}$ substrates. The presence of *β-N*–acetylglucosaminidase activity in the mycorrhizal material was confirmed by use of the chromogenic histochemical stain X-GlcNAc. There was some discrepancy, however, between the results obtained with the two substrates in the extent of *β-N*–acetylglucosaminidase activity in the fungal sheath. Fluorescence of the sheath was not very pronounced using the 4-MU-GlcNAc substrate but there was marked staining by the chromogenic stain X-GlcNAc. This difference may have been due to the soluble 4-MU diffusing away from the site of the cleavage, or, if activity within the sheath was as high as suggested by the X-GlcNAc results, the substrate may have been fully utilized and fluorescence lost by the time of examination. A more detailed study using a range of incubation times might shed further light on this question. The 5-bromo-4-chloro-3-indolyl compound once cleaved becomes insoluble, and loss from the site of cleavage is not possible. This study illustrated the usefulness of this chromogenic substrate in localization studies, particularly when used in conjunction with 4-MU-GlcNAc. The results also suggest the potential of the 4-MU-$(GlcNAc)_{1–4}$ substrates in other plant chitinase localization studies.

References

Agerer R (1987) Colour atlas of ectomycorrhizae. Einhorn, Schwäbisch Gmünd

Albrecht C, Asselin A, Piché Y, Lapeyrie F (1994a) Chitinase activities are induced in *Eucalyptus globulus* roots by ectomycorrhizal or pathogenic fungi during early colonization. Physiol Plant 91:104–110

Albrecht C, Laurent P, Lapeyrie F (1994b) Eucalyptus root and shoot chitinases, induced following root colonization by pathogenic versus ectomycorrhizal fungi, compared on one- and two-dimensional activity gels. Plant Sci 100:157–164

Albrecht C, Burgess T, Dell B, Lapeyrie F (1994c) Chitinase and peroxidase activities are induced in eucalyptus roots according to aggressiveness of Australian ectomycorrhizal strains of *Pisolithus* sp. New Phytol 127:217–222

Boller T (1985) Induction of hydrolases as a defense reaction against pathogens. In: Key JL, Kosuge T (eds) Cellular and molecular biology of plant stress. (UCLA symposia on molecular and cellular biology, vol 22) Liss, New York, pp 247–262

Boller T (1988) Ethylene and the regulation of antifungal hydrolases in plants. Oxford Survey Plant Mol Cell Biol 5:145–174

Boller T, Métraux JP (1988) Extracellular localization of chitinase in cucumber. Physiol Mol Plant Pathol 33:11–16

Brand F (1992) Mixed associations of fungi in ectomycorrhizal roots. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB International, Wallingford, UK, pp 142–147

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3:31–40

Dumas-Gaudot E, Grenier J, Furlan V, Asselin A (1992) Chitinase, chitosanase and β-1,3-glucanase activities in *Allium* and *Pisum* roots colonized by *Glomus* species. Plant Sci 84:17–24

Dumas-Gaudot E, Asselin A, Gianinazzi-Pearson V, Gollotte A, Gianinazzi S (1994) Chitinase isoforms in roots of various pea genotypes infected with arbuscular mycorrhizal fungi. Plant Sci 99:27–37

Gooday GW (1990a) The ecology of chitin degradation. In: Marshall KC (ed) Advances in microbial ecology, vol 11. Plenum, New York, pp 387–430

Gooday GW (1990b) Inhibition of chitin metabolism. In: Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW, Copping LG (eds) Biochemistry of cell walls and membranes in fungi. Springer, Berlin Heidelberg New York, pp 61–79

Gooday GW (1990c) Physiology of microbial degradation of chitin and chitosan. Biodegradation 1:177–190

Graham LS, Sticklen MB (1994) Plant chitinases. Can J Bot 72:1057–1083

Harley JL (1984) The mycorrhizal associations. In: Linskens HF, Heslop-Harrison J (eds) Cellular interactions. (Encyclopedia of plant physiology, vol 17) Springer, Berlin Heidelberg New York, pp 148–186

Hodge A, Alexander IJ, Gooday GW (1995a) Chitinolytic enzymes of pathogenic and ectomycorrhizal fungi. Mycol Res 99:935–941

Hodge A, Alexander IJ, Gooday GW (1995b) Chitinolytic activities of *Eucalyptus pilularis* and *Pinus sylvestris* root systems challenged with mycorrhizal and pathogenic fungi. New Phytol 131:255–261

Leake JR, Read DJ (1990) Chitin as a nitrogen source for mycorrhizal fungi. Mycol Res 94:993–995

Manson FDC, Fletcher TC, Gooday GW (1992) Localization of chitinolytic enzymes in blood of turbot, *Scophthalmus maximus*, and their possible roles in defence. J Fish Biol 40:919–927

McCreath KJ, Gooday GW (1992) A rapid and sensitive micro assay for determination of chitinolytic activity. J Microbiol Meth 14:229–237

Mitchell DT, Sweeney M, Kennedy A (1992) Chitin degradation by *Hymenoscyphus ericae* and the influence of *H. ericae* on the growth of ectomycorrhizal fungi. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB International, Wallingford, UK, pp 246–251

Pierrot H, van Wielink JE (1977) Localization of glycosidases in the wall of living cells from cultured *Convolvulus arvensis* tissue. Planta 137:235–242

Robbins PW, Albright C, Benfield B (1988) Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. J Biol Chem 263:443–447

Roberts WK, Selitrennikoff CP (1988) Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol 134:169–176

Sauter M, Hager A (1989) The mycorrhizal fungus *Amanita muscaria* induces chitinase activity in roots and in suspension-cultured cells of its host *Picea abies*. Planta 179:61–66

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324:365–367

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, van den Elzen PJM, Cornelissen BJC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol 101:857–863

Spanu P, Boller T, Ludwig A, Wiemken A, Faccio A, Bonfante-Fasolo P (1989) Chitinase in roots of mycorrhizal *Allium porrum*: regulation and localization. Planta 177:447–455

St Leger RJ, Cooper RM, Charnley AK (1986) Cuticle-degrading enzymes of entomopathogenic fungi: regulation of production of chitinolytic enzymes. J Gen Microbiol 132:1509–1517

Ulhoa CJ, Peberdy JF (1991) Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum*. Curr Microbiol 23:285–289

Ulhoa CJ, Peberdy JF (1992) Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. Enzyme Microb Technol 14:236–240

Vierheilig H, Alt M, Neuhaus J-M, Boller T, Wiemken A (1993) Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant Microbe Interact 6:261–264

Vierheilig H, Alt M, Mohr U, Boller T, Wiemken A (1994) Ethylene biosynthesis and activities of chitinase and β-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. J Plant Physiol 143:337–343

Wessels JGH, Sietsma JH (1981) Fungal cell walls: a survey. In: Tanner W, Loewus FA (eds) Plant carbohydrates. II. Extracellular carbohydrates. (Encyclopedia of plant physiology, vol 13B) Springer, Berlin Heidelberg New York, pp 352–394