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A HISTOCHEMICAL STUDY OF PHENOLIC MATERIALS IN MYCORRHIZAL AND UNINFECTED ROOTS OF *EUCALYPTUS FASTIGATA* DEANE AND MAIDEN

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SUMMARY

The distribution of phenols in uninfected and mycorrhizal *Eucalyptus fastigata* roots has been examined by application of a range of histochemical tests to thin sections.

The vacuoles of most cap cells and many epidermal cells of *E. fastigata* mycorrhizas contain considerable quantities of phenolic materials. Cap cells of uninfected fine roots similarly contain materials with the staining properties of phenols, but uninfected root epidermal cells rarely do. It is suggested that accumulation of phenols in the epidermal cells of mycorrhizas is a response to the presence of the fungal symbiont. Further evidence is provided that the hyphae of the fungal symbiont penetrate and grow through the phenolic materials of the outer root cap cells. No evidence was found to suggest that these areas contain appreciable quantities of polysaccharide.

Phenols were also detected in the endodermis and outermost layer of cortical cells. Judged on their staining reactions, these seem to be chemically different from those of the cap and epidermis. They are present in both mycorrhizal and uninfected roots, but appear much closer to the tip in the former. The outer layer of cortical cells has other specialized features, including the presence of a suberin layer in the walls, which are characteristic of mature differentiated endodermal cells in *E. fastigata*. These features are discussed from the viewpoint that the outer cortical layer acts as a barrier to further hyphal penetration in eucalypt mycorrhizas.

INTRODUCTION

A prominent but little understood feature of ectomycorrhizas is the accumulation of brown materials within many of the peripheral cells of the host. These materials are usually referred to as 'tannins' and are therefore presumed, on little real evidence, to be phenolic in nature. A range of phenols have been extracted and identified from pine mycorrhizas (Hillis *et al.*, 1968; Hillis and Ishikura, 1969), but it has not been possible to equate any of these with the frequently observed 'brown materials' of the mycorrhizal 'tannin-layer' (Foster and Marks, 1966; Marks and Foster, 1973). Moreover, extractable phenols were also found in uninfected pine roots, albeit in rather lesser amounts. Viewed under the electron microscope (Foster and Marks, 1966), the 'tannin-layer' appears as prominent electron-opaque zones in sections fixed and stained with osmium tetroxide or with potassium permanganate and lead salts. Similar electron-opaque material is also present in some cortical cells of uninfected roots. Marks and Foster (1973) suggested that host tannins might function as a biological screen,

such that only fungi tolerant to these compounds can enter into a symbiotic relationship with the host plant.

Examination of eucalypt mycorrhizas with the electron microscope (Chilvers, 1968) showed osmium tetroxide-stained, electron-opaque material in the vacuoles of root cap and epidermal cells. The deposits were dissimilar in appearance in the two cell types. In epidermal cells it took on a dispersed granular appearance or appeared to precipitate out along the tonoplast, but the material in root cap cells appeared homogeneous throughout. Chilvers (1968) suggested that these materials were tannins. Marks and Foster (1973) commenting on these findings, agreed that the electron-opaque material in epidermal cells (from which the fungus is excluded) was similar to the materials in pine 'tannin-cells' but suggested that the contents of the eucalypt root-cap cells (through which the mycorrhizal fungus was observed to grow quite readily) might be polysaccharide in nature rather than phenolic. The present histochemical study was carried out in an attempt to clarify this point, and to compare the distribution of phenolic materials in mycorrhizas and uninfected fine roots of *Eucalyptus fastigata* Deane and Maiden.

MATERIALS AND METHODS

Mycorrhizas and uninfected roots of *Eucalyptus fastigata* were collected from pot-grown seedlings in spring and early autumn. Most roots were fixed in 8% aqueous acrolein for 20 h at 0°C, then dehydrated, embedded in glycol methacrylate (GMA) and sectioned as described by Feder and O'Brien (1968). Some of the root segments were post-fixed in 10% mercuric chloride before dehydration, to stabilize polyphenols (McCully, 1966). GMA-embedded sections were taken through the following histochemical staining procedures.

Reactions to demonstrate phenols

(a) Toluidine Blue. (Feder and O'Brien, 1968). Sections treated with Toluidine Blue *0* in 0.1 M acetate buffer, pH 4.4, stain a variety of colours, depending upon the macromolecules present. The presence of phenolic substances is indicated by a greenish-blue to green colouration, which persists at low pH (O'Brien, Feder and McCully, 1964; Ramalingam and Ravindranath, 1970).

(b) Ferric chloride. (Johansen, 1940; Mace, 1963 slightly modified). 10% aqueous ferric chloride or 10% ferric chloride in 95% ethanol were flooded onto sections for 10 min or more. Materials containing phenols are reported to stain bluish-colour as a result of the formation of a complex iron salt (Seikel, 1964).

(c) Vanillin test. (Modified from Hawker *et al.* 1972). Sections were dry heated on slides at 150°C for 5 mins before treatment with a freshly saturated solution of vanillin in concentrated HCl. A red colour is produced when aldehyde groups in the vanillin condense with phenols in the tissue.

(d) Millon's reagent. (Modified from Baker, 1956). Sections were heated in an acidified 5% aqueous solution of mercuric sulphate or mercuric acetate for 10 mins at 40°C followed by the addition of 0.5% sodium nitrite. Coloured nitroso derivatives of any phenols contained in the tissues should then become evident.

(e) Hoepfner-Vorsatz reagent. (Reeve, 1951). 2 ml of 10% sodium nitrite were mixed with 2 ml of 10% acetic acid on the section and replaced, after 3 mins, with 4 ml of 2 N sodium hydroxide solution. The action of the nitrous acid on a phenol produces a nitroso derivative which forms a coloured salt on addition of the base.

(f) Gibb's indophenol test. (Zugibe, 1970). Sections were treated for 10–15 min with a 0.1% solution of 2,6-dichloroquinone chloroimide in veronal buffer at pH 9.2. A colour reaction, which may be enhanced by replacing the original solution with 5% NH_4OH and leaving for a further 10 mins to form the blue salt of indophenol, indicates the presence of phenolics or other aromatic ring compounds.

(g) Diazotized p-nitroaniline. (Lillie, 1965). Sections were mounted in a freshly prepared 5 mM solution of diazotized p-nitroaniline in veronal buffer at pH 8.0. The aromatic ring of phenols or aromatic amines couples spontaneously with the diazonium salt to produce a coloured azo dye.

(h) Tetrazotized o-dianisidine. Sections were treated with 0.2% tetrazotized o-dianisidine in veronal buffer of pH 9.2 for 5 min, rinsed briefly 0.1 N HCl followed by water, then post-coupled with a saturated solution of β-naphthol in the same veronal buffer (Burstone, 1955). A brown-coloured azo dye is produced by the above in reaction with phenols or other aromatic ring compounds.

Tests for reducing substances

(i) Ferric-ferricyanide reaction. (Lillie and Donaldson, 1974). Sections were treated with a mixture of 1% ferric chloride and 1% potassium ferricyanide in 2 N acetic acid (pH 2.25) for 5–10 min. In the presence of reducing substances, some of the excess ferric ions will be reduced to the ferrous state, leading to the formation of the blue ferro-ferricyanide (Turnbull's Blue).

(j) Reduction of silver nitrate (Wildi, 1951; Kunoh and Akai, 1969). Sections were covered with Tollen's solution equal volumes of 0.2 N silver nitrate, 2 N ammonium hydroxide and 10% sodium hydroxide) or just 10% aqueous silver nitrate. In both cases, any reducing substances present in the tissue should lead to the formation of a black metallic silver precipitate.

(k) Fehlings reagent. (Kunoh and Akai, 1969). Sections were flooded with a 1:1 mixture of Fehling's solutions A and B, then heated. A brown deposit of metallic copper indicates the presence of reducing substances.

Reactions to demonstrate protein

(l) Acid Fuchsin. (Feder and O'Brien, 1968). Sections were stained with 0.005% acid fuchsin in 1% aqueous acetic acid for 10 mins to demonstrate proteins. This provides a useful test to discriminate between phenols and aromatic amino acids, since only the latter stain.

(m) Naphthol Blue Black. (Fisher, 1968). Sections were stained in 1% Naphthol Blue Black in 7% acetic acid for 10 mins at room temperature, and then either rinsed with 7% acetic acid followed by distilled water or distilled water alone. This staining procedure, based on that used by Fisher for Epon sections, also gives excellent results with GMA sections.

Reactions for carbohydrates

(n) Periodic Acid-Schiff Stain. The PAS procedure for the detection of carbohydrates was carried out as described by Feder and O'Brien (1968), except that pre-treatment with 2,4-dinitrophenylhydrazine was extended to 2 h in order to completely block the aldehyde groups already in tissue prior to periodate oxidation. Some sections were pre-treated with concentrated bromine solution (Jensen, 1962) to block any unsaturated double bonds in lipids which might break to form aldehyde groups under the influence of the periodic acid oxidation and hence give a false positive reaction with the Schiff's reagent.

Areas of the tissue which stained in the PAS reaction after blockade with either 2,4-dinitrophenylhydrazine or bromine, but which did not stain in controls (without periodate oxidation) were considered to be PAS positive. Further proof that 1:2 glycol groups are involved in the PAS reaction was obtained by pre-treatment of sections for 45 mins with acetic anhydride (13 ml acetic anhydride in 20 ml pyridine), which block the hydroxyl groups (McManus and Cason, 1950).

(o) Alcian Blue. (Lev and Spicer, 1964). Sections were stained with Alcian Blue 8GX at either pH 2.5 or pH 1.0, according to the method of Lev and Spicer (1964). At pH 2.5 both carboxyl and sulphate groups of acid mucopolysaccharides stain but at pH 1.0, which is below the pK of carboxyl groups, only sulphated polysaccharides stain.

Lipid stain

(p) Sudan black B. (Pearse, 1968). Sections were stained for 5 minutes in a saturated solution of sudan black B in 70% ethanol, rinsed briefly in 70% ethanol, and mounted in glycerol-gelatin.

Fluorescence microscopy

Fluorescence microscopy was used to investigate the autofluorescence of the phenols present. Unstained sections were viewed and photographed by incident light fluorescence microscopy, using a Zeiss Universal Microscope, with an HBO 200 W/4 lamp and various exciter and barrier filter combinations. The best results were obtained with the following filter combination: UG1 UV-transmittent black glass, FT 420 chromatic splitter and LP 418 colourless UV barrier filter.

RESULTS

The contents of fungal hyphae and epidermal cells of the host were generally well preserved in both of the fixation and embedding procedures used, but the cells of the mycorrhizal cortex, particularly those of the outer cortex, were difficult to fix and were frequently damaged. A few roots failed to impregnate properly in the stelar region. Post-fixation with

$HgCl_2$, after acrolein fixation, did not change either the appearance of the tissues or the histochemical reactions.

Most of the histochemical procedures gave satisfactory colour reactions when applied to GMA-embedded sections. Exceptions were Millon's reagent *(d)* and Gibb's indophenol test *(f)* which gave only very weak colours and consequently results of doubtful value. Some of the other tests were quite destructive of the sections (e.g. vanillin *(c)*, Fehlings reagent *(k)*) which were consequently difficult to photograph satisfactorily. The specific observations are divided into sections below, according to the tissues examined.

Root cap cells

Mycorrhizas. The cap cells of mycorrhizas contained large quantities of material which stained a strong greenish-blue with Toluidine Blue at pH 4.4 (Plate 1, Nos. 1 and 2). This colour persisted at pH 1.0 when the purple staining of the cytoplasm-filled hyphae and meristematic cells was totally suppressed (Plate 2, No. 10). The appearance of the material varied in different regions of the root cap. In the inner cap cells it was distinctly located in the vacuoles and often had a dispersed finely granular appearance (Plate 1, No. 2), while in the outer cap cells it was much more homogeneous and filled most of the cell lumen. The intensity of staining with Toluidine Blue in the outermost cap cells was greater than in the inner cap cells. There were a few exceptions where the inner cap cells had contents more characteristic of outer cap cells and *vice versa*, and also in a few cases the material of 'homogeneous' appearance was rounded up into globules of various sizes.

The material which stained with Toluidine Blue, also stained in six out of seven other histochemical tests *(b–h)* sensitive to phenols or other aromatic ring compounds. The exception was Millon's reagent *(d)*, which was judged to be an unsatisfactory test anyway. The response to the Hoepfner-Vorsatz test *(e)*, is shown in Plate 2, No. 5. The contents of many of the cap cells were also quite reactive in tests *(i–k)* indicating the presence of reducing substances. The staining resulting from the reduction of silver nitrate is shown in Plate 2, No. 7. The staining in the root cap cells was not uniform. The contents of inner cap cells stained a yellowish colour, which was not much different from staining in the cytoplasm of the root meristematic cells, while the homogeneous material in outer cap cells stained brown. This implies that the capacity of the phenolic material to act as a reducing substance is greater in the outer cap cells. Such differences in intensity of staining between contents of inner and outer cap cells were also obtained with some of the other staining reactions. With Reeve's Hoepfner-Vorsatz reaction, shown in Plate 2, No. 5, the contents of the outer cap cells again stained more intensely than inner cap cell contents, while with tetrazotized o-dianisidine, on the other hand, staining of the inner cap cell contents was greater than that of the outer cap cell contents (Plate 2, No. 8). The cap cell contents did not react with protein stains *(l–m)* indicating that aromatic amino acids are not responsible for the positive staining reactions indicating phenols (Plate 2, No. 9). Penetration of the phenolic material in the outer cap cells by fungal hyphae was commonly observed in the mycorrhizas examined (for example see Plate 1, No. 2, and Plate 2, Nos. 5 and 10).

Reactions with various carbohydrate stains indicated that the vacuolar contents of the root cap cells did not contain appreciable amounts of polysaccharide. With PAS reagents the reactions were complex. The phenol itself is very Schiff positive (i.e. it stains with Schiff's reagent without periodate oxidation). The Schiff-positive groups are very difficult to block and require at least 2 h treatment with 2,4-dinitrophenylhydrazine for complete blockade.

A further complication was that in incompletely blocked sections, the intensity of staining increased after periodate oxidation. The basis of this is not understood, but since this only occurs in incompletely blocked material, it must be assumed to be due to an increase in staining of Schiff-positive groups already present, rather than introduction of new aldehydes from 1:2 glycol groups in polysaccharides. After adequate blockade, treatment with periodic acid did not introduce new aldehyde groups from oxidation of 1:2 glycols, and the vacuolar material often stained a yellow to reddish brown colour following the PAS reaction, presumably as a result of oxidation of the phenol, but this colour was readily distinguished from the magenta colour of the complex formed between Schiff's reagent and free aldehydes. This indicates that the vacuoles do not contain neutral polysaccharides in significant quantities. It also illustrates the need for extreme care in interpreting the PAS reaction.

Not all polysaccharides are PAS-positive. Characteristically, many acid mucopolysaccharides however, also gave negative results. With basic dyes, such as Toluidine Blue, acid groups of polysaccharides stain metachromatically. Although this reaction is often masked when phenols are present, it may be enhanced so that it is visible even in the presence of phenols by mounting the sections in water. There was no evidence of pink metachromatic staining, even in water-mounted sections. Similarly, the vacuolar contents did not normally stain with alcian blue. It is unlikely that a polysaccharide would be present in any quantity and not stain in one or other of these tests. Finally the vacuolar contents were not extracted with any of a wide range of carbohydrate-digesting enzymes which were applied to mycorrhizal sections, although some of these enzymes removed all the starch and cell wall material from the section.

There was a well-developed layer of phenol-staining material in the inner region of the fungal sheath, just external to the epidermal cells of the root (Plate 1, No. 1; Plate 3, Nos. 11–16). At low magnification, the layer appeared to be more or less continuous over the root surface (Plate 1, No. 1), but at higher magnifications it was seen to consist of discrete areas of phenol-staining material, through which the fungus was growing (Plate 3, Nos. 11–16). The staining (in tests *a–k*) and physical appearance of material in these areas was similar to that in the outer cap cells of mycorrhizas and all the cap cells of uninfected roots, except that in most tests the areas stained more intensely than the outer root cap cells, e.g., with Toluidine Blue (Plate 1, No. 2), tetrazotized o-dianisidine, and the silver nitrate reaction. The appearance, staining and shape of these areas, and their continuity with the root cap, suggested that most of them were the remains of outer root cap cells in the process of being disorganised.

Uninfected roots. The root caps of uninfected fine roots were very much larger than mycorrhizal root caps, there being many more layers of cells in the cap (compare Plate 1, Nos. 1 and 2 with Nos. 3 and 4). Almost all of the cap cells had contents which stained in a similar way to the mycorrhizal cap-cell contents already described, in all fourteen tests. In most cells the material could be seen to be located in the cell vacuoles, and as in mycorrhizas its appearance varied from cell to cell (Plate 1, No. 4). In general, however, material of homogeneous appearance, similar to that seen in outer cap cells of mycorrhizas, predominated and there was no clear-cut distinction between vacuolar contents of inner and outer cap cells as in mycorrhizas. Results with a range of staining reactions *(a–k)* suggested that the compounds in the vacuoles of uninfected root cap cells were similar to those in mycorrhizal cap cells and were phenolic in nature (e.g. compare Plate 1, Nos. 2 and 4, and Plate 2, Nos. 5 and 6). As in mycorrhizas, the vacuolar contents did not stain with protein or carbohydrate

stains *(l–o)*, indicating that neither protein nor polysaccharide was present in histochemically detectable amounts.

Remains of collapsed root cap cells showing histochemical reactions for phenols were found adhering to the root surface at quite considerable distances from the root tip.

Epidermal cells

Mycorrhizas. The radially elongated epidermal cells of many mycorrhizas also contained material in their vacuoles which stained a greenish-blue colour with Toluidine Blue, with or without an acid rinse (Plate 3, Nos. 11 and 12). These had the same dispersed granular appearance as the electron-opaque osmium-stained material in epidermal cells viewed in the electron microscope, previously described by Chilvers (1968). The appearance of these vacuolar contents varied considerably in different mycorrhizas, and in some they were absent altogether. The reason for this is not certain, but from the range of mycorrhizas studied it did seem that the presence of such materials was typical of actively growing mycorrhizas collected during the period of peak growth, and that they were absent in apices collected at times of limited growth.

As well as their greenish-blue acid-persistent staining with Toluidine Blue, the materials in the epidermal cell vacuoles also gave positive reactions in the various other histochemical tests for phenols *(b–h)* and in addition were shown by tests *(i–k)* to be reducing substances. They did not react with protein stains *(l–m)* and their staining reactions with carbohydrate stains *(n–o)* were identical to those of the root cap vacuolar contents so it is concluded also that they do not contain polysaccharide.

Uninfected roots. The epidermal cells of uninfected roots did not show the strong positive reaction to tests for phenolic compounds that characterized the epidermal cells of mycorrhizas. In young epidermal cells, at least up to 2 mm from the root tip (an equivalent distance from the tip to the region examined in mycorrhizas), there was no evidence that phenols were present. Traces of phenols are eventually accumulated in the vacuoles in much older epidermal cells (e.g. in regions about 10 cm from the root tip) but their appearance is quite different from phenols induced in the mycorrhizal epidermis (Plate 4, No. 17). The walls also become lignified in older epidermal cells and eventually they collapse.

Outer cortical cells

Mycorrhizas. The outer layer of cortical cells in ***Eucalyptus fastigata*** mycorrhizas is a single layer of cells which is differentiated both morphologically and histochemically from the other cells of the root. This layer has been referred to as an 'exodermis' or 'hypodermis' (see Esau, 1965, p. 487). The staining characteristics of the walls of many cells in this layer indicated that they are quite specialised in comparison with the inner layers of the cortex. For instance, in differentiated cortical cells near the tip of slower growing mycorrhizas, a very thin layer in the region of the wall around each cell stained blue with Toluidine Blue suggesting that phenols are present. This is difficult to distinguish from the very heavily stained cell contents described below, except in cells which have been damaged during fixation and embedding, where the cell contents have contracted away from the walls (Plate 4, No. 15). Similarly, a thin layer in the wall around each outer cortical cell stained black with Sudan Black B indicating that a lipid layer was present (Plate 4, No. 20). This suggested

that there was a 'suberin' layer in the walls of many of the outer cortical cells in this region of the root. The layer was continuous around the entire cell but its exact location in the wall in relationship to the phenol-staining layer could not be resolved in the light microscope.

Most outer cortical cells also had contents which gave a positive reaction in some of the tests for phenols. With Toluidine Blue at pH 4.4, a band of material around the cell periphery stained deep blue, which was quite different from the greenish-blue staining of the root-cap phenols embedded in the sheath (Plate 3, No. 15 and Plate 4, No. 19) or the overlying epidermal cells when these also contained phenols. The outer cortical cells were generally poorly fixed and in most cases cytoplasmic material could not be resolved from material around the periphery of the vacuole (e.g. Plate 3, No. 15; Plate 4, No. 18). In a few less poorly fixed cells the phenol-staining material was predominantly in the vacuole, precipitated as a thin layer around the tonoplast. It seems most likely that during fixation the phenol-staining materials have leaked from the vacuole and become absorbed throughout cytoplasm, producing secondary staining of cytoplasm and nucleus.

The band of material which stained blue with Toluidine Blue at pH 4.4 retained its blue colour at low pH (Plate 4, No. 19). It also gave good positive reactions with several of the other histochemical tests for phenols. However, differences in colour and degree of staining in many of these reactions suggested that the material has a different chemical nature from the phenolic compounds in the cap and epidermal cells. For instance it gave stronger colours with azo-coupling reactions *(g* and *h)* and in two of the reactions for reducing substances *(i* and *j)*. In addition it has a strong reducing action on osmium tetroxide, and a very high affinity for lead salts, as shown in the lead sulphide reaction (see Plate 1, No. 3, Ashford, Ling-Lee and Chilvers, 1975). On the other hand the material did not stain in tests *b*, *c*, and *e*. There was no reaction with either of the protein stains *(l* and *m)*, nor with the PAS procedure *(n)*. The material did, however, give a strong staining reaction with Alcian Blue *(o)*. The significance of this is pointed out below.

In the outer cortical cells described above, the materials giving a positive reaction with Toluidine Blue occurred exclusively around the periphery of the cell; central regions of the vacuole were free of staining (Plate 3, No. 15; Plate 4, Nos. 18 and 19). Outer cortical cells of this description were characteristically found in slow-growing mycorrhizas (where the epidermal cell vacuoles were free of phenols). In contrast, in actively growing mycorrhizas, (which contained large amounts of phenolic material in epidermal cell vacuoles), the vacuoles of many of the outer cortical cells were filled with a diffuse material which gave staining reactions characteristic of an acidic compound, most probably a polysaccharide. The material stained pale purple with Toluidine Blue, pH 4.4, (Plate 3, No. 11) and blue with Alcian Blue, pH 2.5. Both of these colour reactions were suppressed at low pH. Cells containing this diffuse material sometimes had the blue-staining band of material around the cell periphery (Plate 3, No. 11), but more often lacked this feature. It appears likely that the Alcian Blue reaction of the diffuse material and the peripheral band are due to one and the same polysaccharide material, distributed throughout the vacuole on some occasions and precipitated out along the tonoplast in others. The latter phenomenon appears to relate to the presence of phenols. A combined precipitate of polysaccharide and phenols would explain well the particularly intense blue colour developed with Toluidine Blue, which could result from the additive effects of the purplish-blue metachromasy developed with the acidic polysaccharide and the greenish-blue metachromasy developed with phenols. Ramalingam and Ravindranath (1970) noted a similar blue reaction with artificial mixtures of mucopolysaccharides and phenols.

Uninfected roots. In uninfected roots, at a similar distance back from the tip as the outer cortical cells examined in mycorrhizas, the outer cortical layer of uninfected roots was in very early stages of differentiation and did not show any of the special features of this layer described in mycorrhizas. However, in older, more differentiated, regions of the roots, as shown in Plate 4, Nos. 17 and 21, the outer cortical cells have very similar features to the outer cortical cells of mycorrhizal roots. Staining with Sudan Black B indicated that a thin lipid layer was present in the wall (Plate 4, No. 21). Similarly, staining with Toluidine Blue pH 4.4 showed a narrow bright-blue band around the cell periphery, which could be resolved in some plasmolysed cells into a band in the inner wall region and a band associated with the cytoplasm (Plate 4, No. 17). In addition, an acid polysaccharide with identical staining reactions to that in many of the outer cortical cells of fast growing mycorrhizas, also occurred in outer cortical cells of uninfected roots (compare Plate 4, No. 17 with Plate 3, No. 11).

Inner cortical cells

Chilvers and Pryor (1965) drew attention to the thickening of radial and inner tangential walls of the inner cortical cells of differentiated regions of eucalypt mycorrhizas. With Toluidine Blue, these walls stained pale purple in regions approximately 2 mm from the tip, but further back along the mycorrhiza they stained pale green, suggesting that phenols, probably lignin, had been incorporated into the cell wall. Chilvers and Pryor (1965) did not find these in uninfected roots and so concluded that they were a special feature of mycorrhizas. However, further examination of more mature regions of a range of uninfected roots has shown that the inner cortical cells of these also possess similar thickening of the inner tangential and radial walls (Plate 4, No. 17). Frequently the outer tangential walls were also thickened as well.

The Endodermis

In the region examined in greatest detail (approx. 2 mm from the root tip) the endodermis in mycorrhizas comprised a mixed population of cells. In some cells there was a distinct Casparian strip in the radial walls, while other cells were surrounded by a continuous thin layer of lipid-staining material (stained with Sudan Black B) suggesting that a suberin lamella was present in the walls (Plate 4, No. 20). Most of the endodermal cells were filled with materials with the staining properties of phenols (Plate 4, No. 19) but the physical appearance and staining of this material was different in different cells. In cells with Casparian strips, the phenolic material was similar in staining characteristics to that in epidermal cell vacuoles (compare Plate 4, No. 19 with Plate 3, No. 12). It was homogeneous or very finely granular, and stained a pale green-blue colour with Toluidine Blue pH 4.4. This material filled the entire vacuole. In cells with suberin lamellae the phenolic material was mostly precipitated at the tonoplast in a thick homogeneous band. With Toluidine Blue, both at pH 4.4 and 1.0, this band stained a similar deep blue to the band of phenolic material around the cell periphery of the outer cortical cells (Plate 4, No. 19). There was also similarity in the staining of both of these bands in other tests. In older regions of mycorrhizas a greater proportion of the endodermal cells were of the second type and in many of these there was evidence also that the purple staining acid polysaccharide, characteristic of outer cortical cells, was beginning to accumulate.

The presence of phenols in the vacuoles of endodermal cells is not an exclusive feature of mycorrhizal roots. In regions of uninfected roots, an equivalent distance from the root tip

of the regions examined in mycorrhizas, the endodermis was only in early stages of differentiation, and the cells did not contain phenol-staining materials. However, in older, more differentiated regions, most of the endodermal cells had developed a suberin lamella (Plate 4, No. 21). Phenolic material was also precipitated in a band along the tonoplast in many of these cells, and in addition a diffuse purplish staining, indicative of the presence of an acid polysaccharide, also occurred throughout the vacuole. Thus, in staining properties and appearance, the contents of these endodermal cells were very similar to the contents of mature endodermal cells of mycorrhizas which had already developed a suberin lamella in their walls (also to the contents of most outer cortical cells in this region of uninfected roots, and of some outer cortical cells in mycorrhizas).

Fluorescence microscopy

Data from fluorescence microscopy supported the observations with other stains. With the filter combination described in the methods section, the residual root cap cells some distance back from the cap fluoresced a bright salmon colour, which was identical to the fluorescence of the phenols in outer cells within the cap itself. This could be distinguished readily from the deeper orange fluorescence of the epidermal cell vacuolar contents, when present, and the weak yellowish-green fluorescence of the contents of some outer cortical cells. In contrast to the yellow fluorescence inside the cells, the walls of outer cortical cells fluoresced a bright blue, an identical colour to the fluorescence of xylem thickenings in the same section. Thus, the phenols located in different areas of the section all exhibit characteristic and different fluorescence colours, suggesting that there are at least minor differences in their chemical composition. A photomicrograph of fluorescence in the outer layers of a mycorrhizal root section is shown in Plate 3, No. 16. There is also fluorescence in the fungal cell walls, for example in the Hartig net region, but this has not been investigated further.

DISCUSSION

This appears to be the first time that the distribution of phenols in mycorrhizas has been examined by histochemical methods. The histochemical approach has been successful in that it has given information about the phenol content of individual cells in different tissues of uninfectedand mycorrhizal roots, information which is not available from classical extraction and analysis procedures.

While individual staining reactions are not always specific enough by themselves to give unequivocal evidence of the presence of phenols, a positive result with several different tests can be taken as good evidence that phenols are present in that location. This criterion was readily satisfied throughout the present study and it is clear that phenols are widely distributed through the tissues of both mycorrhizas and uninfected roots of *Eucalyptus fastigata*.

In mycorrhizas, phenols are present in the vacuoles of root cap cells, epidermal cells, outer cortex, endodermis, some cells of the stele, and also as deposits embedded in the fungal sheath, derived from root cap cells. With the prominent exception of those in the epidermal cell vacuoles, these phenols do not seem to be induced by infection with the fungus, as they are present in the same tissues of uninfected roots. However, in mycorrhizas phenols appear right up to the root tip in all the above tissues, whereas in uninfected roots, with the exception of the root cap itself, they do not start to build up until some considerable distance back from the tip, so that the root tip region is relatively free of phenols. This difference seems to be based on differences in the relative rates of growth and differen-

tiation: in the slower growing mycorrhizas differentiated tissue extends closer to the root tip than in uninfected roots.

The staining properties of phenols in tissues of mycorrhizas are invariably the same as those of phenols in the same tissues of uninfected roots. In contrast, phenols in different tissues of the same root, whether uninfected or mycorrhizal, gave different staining reactions. Thus, while there was a great deal of similarity among the staining reactions of phenols in the outer root cap cells, the residues of root cap cells embedded in the mantle and in the epidermal cells of mycorrhizas; phenols of outer cortical and some endodermal cells gave quite a different set of reactions to these.

Although we may conclude that phenolics which stain differently are likely to be chemically different, the reverse is not necessarily true. Most of the staining reactions used in histochemistry indicate only broad similarities in chemical structure, such as the presence of a phenol ring or of strongly reducing groups. Therefore when staining reactions are the same it cannot necessarily be assumed that the compounds will be chemically identical. This argument can be applied to the comparison between the phenols in the root cap and epidermal cell vacuoles. Thus, while their staining reactions are very similar, there are subtle differences in fluorescence emission (salmon-pink versus orange colour) which suggests the possibility that there might be slight differences in chemical composition. The vacuolar materials of root cap and epidermal cells also differ somewhat in physical appearance (homogeneous versus granular deposits), a point made previously by Chilvers (1968) on the basis of electron microscope observations, and recalled by Marks and Foster (1973) when criticising his suggestion that these were both 'tannin-like' (i.e. phenolic) materials. They emphasized, quite correctly, that electron opacity following osmium tetraoxide and uranyl ion treatment is a rather non-specific staining reaction that does not of itself prove that these materials were phenolic, and they offered an alternative view that the electron dense materials of the eucalypt root cap were quite different from those of the epidermal cells, being polysaccharides rather than phenols. The present study strongly reaffirms the earlier view (Chilvers, 1968), since both the root cap and epidermal materials gave strongly positive results with a number of histochemical tests for common classes of polysaccharide. If any polysaccharides are present in these vacuolar materials at all, they must occur there at quite low concentrations.

Comparisons between the histochemical properties of the phenolic materials embedded in the inner sheath (the 'tannin layer' of other authors, e.g. Marks and Foster, 1973) and those of the material filling the outer cap cells, have lent support to the contention (Chilvers, 1968) that the former are derived from the latter. The pattern of histochemical reactions and the fluorescence colour is identical for both, and in median longitudinal sections stained for phenols the cap tissue at the tip is clearly continuous with the layer of phenolic deposits running back along the flanks of the mycorrhiza. Based on observations with the electron microscope, Chilvers (1968) concluded that hyphae of the inner sheath grow through and subdivide the cap cells to produce these fragmentary residues. With the contrasting staining produced by Toluidine Blue (pale-purple hyphae compared with greenish-blue cap cell contents) fungal penetration of the cap cells and these residues was observed frequently in the present study. The fact that this occurs is significant. Phenolic materials are frequently implicated in defence mechanisms of plants against invading fungi (Kūc, 1972) but the evidence from the present paper and the earlier study by Chilvers (1968) suggests that the phenolic materials in the root cap cells do not offer any resistance to invasion by the mycorrhizal fungi of eucalypts. This does not, however, rule out the suggestion made by Marks and

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EXPLANATION OF PLATES

PLATE 1

Longitudinal sections through mycorrhizal and uninfected root tips stained with Toluidine Blue at pH 4.4 (Tip orientated towards top of page).

No. 1. Mycorrhizal root showing general distribution of greenish-blue stained phenols in the tip region (× 350). RC = outer layers of root cap, T = phenol ('tannin') layer in inner sheath region, M = purple stained meristem, St = developing stele. Near the apex, the phenolic material in some outer cap cells has shrunk during embedding, leaving colourless gaps between cell contents and walls.

No. 2. Portion of mycorrhizal cap region magnified further to show the different appearance of phenols in different layers (× 1100). S = fungal sheath tissue, T_1 = granular phenols dispersed through vacuoles of inner cap cells, T_2 = homogeneous phenols apparently filling the lumen of outer cap cells, T_3 = more intensely stained phenols of cap cells or residues of cap cells isolated within the sheath. The asterisk indicates one of three hyphae which have penetrated an outer cap cell.

No. 3. Uninfected root tip showing general distribution of phenols (× 180). M = meristem, T = intensely stained phenols in the vacuoles of peripheral cap cells (the white tipped arrows point to flattened, drawn out cap cells on the flanks).

No. 4. Portion of uninfected cap region at higher magnification (× 950). T = homogeneous phenolic contents of cap cells, arrows = nuclei and cytoplasmic lining of cap cells distinguished by a purple staining reaction from the greenish-blue stained phenols, asterisks = unevenly precipitated phenols of some leading cap cells, P = polysaccharide slime.

PLATE 2

Root-cap cells near to tip of mycorrhizas and an uninfected root, variously stained to demonstrate presence of phenols (× 950). (Tip orientated towards top of page; M = meristem, S = sheath.)

No. 5. Mycorrhizal root cap tissue stained with Hoepfner-Vorsatz reagents. RC = inner cap cells with stained vacuolar phenols. Unstained fungal tissue (asterisks) show up clearly where they grow through the more deeply stained vacuolar contents of outer cap cells.

No. 6. Uninfected root cap tissue stained with Hoepfner-Vorsatz reagents. T = deeply stained phenolic contents of the cap cell vacuoles.

No. 7. Mycorrhizal root cap tissue stained with silver nitrate solution. The contents of most cells, including those of the inner root cap (RC), stained a non-specific dull yellow colour, but the outer cap cells stained brown, indicating a greater reduction potential.

No. 8. Mycorrhizal root cap tissue stained with tetrazotized o-dianisidine. Contents of the inner cap cells and a thin band around the periphery of the outer cap cells stained red. Meristematic cells and the remainder of the outer cap cells stained yellow.

No. 9. Mycorrhizal root cap tissue stained with Naphthol Blue-Black—a protein stain. In contrast to the dark hyphal and meristematic cell contents, the root cap cell contents (RC) are completely unstained.

No. 10. Mycorrhizal root cap tissues stained with Toluidine Blue at pH 1.0. Greenish-blue staining of the cap cell phenolics persisted but purple cytoplasmic staining was suppressed,

compared with the same stains at pH 4.4. Asterisks indicate unstained fungal hyphae penetrating through cap cell phenolic material.

PLATE 3.

Median longitudinal sections of the epidermal and outer cortical region of mycorrhizas, approximately 1 mm back from the apex, stained in various ways to demonstrate the presence of phenols (× 950). (Orientated with tip towards left, fungal sheath towards top, of page). E = epidermal cell, C = outer cortical cell.

No. 11. Section of an active mycorrhiza stained with Toluidine Blue at pH 4.4. Granular material dispersed through vacuoles of epidermal cells stained greenish-blue, indicating phenols. Vacuoles of most outer cortical cells were filled with a diffuse, purple-staining material and bordered by a dark blue stained band around the periphery.

No. 12. Section of an active mycorrhiza stained with Toluidine Blue at pH 1.0. Phenolic materials of root cap residues, epidermal vacuoles, periphery of the outer cortical cells, and vacuole of the endodermis (En), all stained greenish-blue or blue; no purple staining was evident. The outer cortical cells were distorted and poorly fixed in this section. Asterisks = hyphae within root cap residues.

No. 13. Section of an active mycorrhiza stained with silver nitrate. Epidermal cell vacuolar contents and root cap residues embedded in the sheath (arrows) stained brown.

No. 14. Section of active mycorrhiza stained with Hoepfner-Vorsatz reagents. Root cap residues and vacuolar contents of epidermal cells stained red; outer cortical cell contents did not.

No. 15. Section of slow growing mycorrhiza stained with Toluidine Blue at pH 4.4 (compare with No. 11). There were no phenols in the epidermal cell vacuoles and no diffuse purple staining material in the vacuoles of outer cortical cells, but there was a peripheral band of dark blue staining (arrows) involving the cytoplasm and nucleus (n).

No. 16. Fluorescence micrograph of a similar region to No. 15, taken with UG1, FT 42 and LP 418 filter combination and an HBO 200 U.V. lamp. The root cap residues fluoresced a salmon colour, the hyphal walls of the Hartig net a yellow colour, the walls of the outer cortical cells a bright blue, and there was a weak yellow-green fluorescence throughout the vacuole of some outer cortical cells. The epidermal cells of this section contained no vacuolar phenols; where these are present they fluoresce deep orange.

PLATE 4.

Transverse sections of mycorrhizal and uninfected roots to illustrate the similarities between the endodermis and outer cortex in both. (E = epidermis, C = outer cortex, Ci = inner cortex, En = endodermis.)

No. 17. Section of an uninfected root taken several mm back from the tip, stained with Toluidine Blue at pH 4.4 (× 650). Most outer cortical cells show diffuse (purple) staining throughout the vacuole; one which does not, has a band of dark blue stained material associated with the cytoplasm which has plasmolysed away from the wall (small arrows). Some endodermal cells (En) have purple stained material throughout the vacuole and a dark blue stained band around the periphery of the cell.

No. 18. High power view of outer cortical cells of mycorrhiza stained with toluidine blue at pH 4.4 (× 1600). At the points indicated by the two arrows, the cytoplasm has torn away from the cell wall. Dark (blue) stained phenols can be seen to occur both in this cytoplasm and as a thin band within the inner wall region.

No. 19. Section of a mycorrhiza stained with Toluidine Blue at pH 1.0 (× 650). Most outer cortical cells and half of the endodermal cells (arrowed) had a dark blue stained band of material around the periphery. Other endodermal cells (asterisked) contained fine granular vacuolar contents which stained a pale greenish-blue. One endodermal cell and one outer cortical cell (C_o) did not give any reaction for phenols. T = phenolic material of root cap residues.

No. 20. Another section of the same mycorrhiza taken close (8 μm away) to that shown in

No. 19 above and stained with Sudan Black B (× 750). A stained layer of lipid material occurs in the wall of most outer cortical cells and half of the endodermal cells (arrowed). Other endodermal cells (asterisked) appeared to have Casparian strips but these do not show up in this photograph. The same cells appear in the endodermis shown in No. 19 and No. 20, and are similarly labelled to facilitate comparison. Note that most of the cells with a lipid wall layer also have a peripheral band of phenolic material.

No. 21. Section of uninfected root stained with Sudan Black B (× 750). Outer cortical cells and most endodermal cells had a stained layer of lipid material in the walls (arrows).