ULTRASTRUCTURAL OBSERVATIONS ON PUCCINIA MENTHAE INFECTIONS

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SUMMARY

Haustoria and associated regions of the host-pathogen interfaces of the autoecious rust Puccinia menthae, during mono- and dikariotic phases of development on its host Mentha spicata, differed in ultrastructural and cytochemical features. By application of the PA-TCH-SP procedure, polysaccharides were shown not only to be major constituents of the walls of intercellular hyphae, haustorial mother cells (HMC) and haustoria of both the monokaryon and dikaryon (m- and d-haustoria, respectively) of P. menthae, but were also present within the extra-haustorial matrix (EM) of both types of haustoria. In the neck region of d-haustoria, which is structurally more differentiated than in m-haustoria, there was evidence for the presence of lipopolysaccharides and glycoprotein. However, the characteristic neck-band, which stains densely in conventional staining of the d-haustorium, did not react with PA-TCH-SP. The outermost layer of the dikaryotic HMC wall was completely digested by protease, whereas treatment with protease and cellulase did not affect either mono- or dikariotic intercellular hyphae or walls of m-haustoria, although these treatments indicated the presence of protein and cellulose in the EM. In dikaryotic infections, protein was found to be a major constituent of the EM. Cellulase treatment resulted in almost complete digestion of the host wall, except for a thin outer layer, whereas hyphal and haustorial walls were unaffected. The morphology and structural differences observed between the mono- and dikaryotic infection structures of P. menthae, and their interactions with host cells were clarified, and confirmed the possibility of their functional differences. Such information should facilitate further comparative studies on host-parasite interfaces at different stages of the rust life cycle and other biotrophic fungi.

Key words: Puccinia menthae, rust haustoria/host interfaces, EM-cytochemistry, cytopathology.

INTRODUCTION

Ultrastructural investigations of a wide range of rust infections have revealed that haustoria formed during the monokaryotic (m-) phase of the life cycle are of less specialised structure than those of dikaryotic stages, regardless of whether developing in the same or different host species, i.e. autoecious or heteroecious rusts (Harder, 1978; Littlefield and Heath, 1979; Al-Khesraji and Lösel, 1981; Mims, 1991; Baka and Lösel, 1992; Larous and Lösel, 1993a). Relatively little is known about the chemical composition of the macromolecular components of m- and d-haustoria. The most thoroughly investigated rusts in this respect are the heteroecious spp. Puccinia coronata and Puccinia graminis (Chong et al., 1981, 1986; Marticke et al., 1998). Cytochemical methods for detection at ultrastructural level of certain chemical components, including the selective removal of specific substances such as carbohydrates, proteins and cellulose by respective enzymes, have been used to characterize the host-parasite interactions of various groups of fungi (Chong et al., 1981; Coffey and Allen, 1983; Chong et al., 1986; Marticke et al., 1998; Hu and Rijkenberg, 1998).

The autoecious, macrocyclic rust P. menthae, which occurs commonly as a systemic infection of shoots and rhizomes of M. spicata, was selected for a comparative ultrastructural and cytochemical study of haustoria and associated interfaces, in order to gain a better understanding of the interaction of this pathogen with the host, throughout its life cycle, without the physiological complications of heteroecious rusts, where mono- and dikaryotic phases occur on separate host species. Some information on the pathogenicity, phylogeny, physiology and control of P. menthae is available (Baxter and Cummins, 1953; Van Der Merwe et al., 2007; Edwards et al., 1998) but, apart from our previous investigation of vascular infection by this rust (Larous and Lösel, 1993a), and of the distribution of ATPase at its host-pathogen interfaces (Baka et al., 1995), no ultrastructural or cytochemical investigations of this pathogen appear to have been published.
MATERIALS AND METHODS

Plant material. Systemically infected plants of garden mint, bearing pycnia and aecia of *P. menthae*, were collected from the field in late spring. Uredinal and telial stages were obtained by inoculation of the leaves of plants grown in growth-room conditions (20±2°C, 80 Watts m², 16 h photoperiod) with aeciospores, as previously described (Larous and Lösel, 1993a).

Conventional fixation and staining for electron microscopy. Infected tissue was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer then, either after postfixation with 1% osmium tetroxide for 2 h or without postfixation, was dehydrated by graded ethanol dilutions and embedded in Araldite (Larous and Lösel, 1993a). Ultra-thin sections were cut with a diamond knife, post-stained with 2% aqueous uranyl acetate followed by lead citrate (Reynolds, 1963), and examined with a Philips 301 electron microscope (ca. 50 blocks of 1 mm³ of different infected mint leaves were treated).

Cytochemistry. The periodic acid/thiocarbohydrazide/silver proteinate procedure (PA-TCH-SP) of Thiery (1967) was used to detect polysaccharides with vicinal hydroxyl groups. For enzymic digestion, glutaraldehyde-fixed tissue was exposed to protease (Sigma, type XIV), 5mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, or to cellulase (Sigma, type I, practical grade), 5mg/ml in 0.05 M phosphate buffer, pH 5.5, for 16-24 h at 37-40°C, before postfixation with osmium tetroxide (Hickey and Coffey, 1978). For control treatments, the tissue was incubated in the corresponding buffer in the same conditions.

RESULTS AND DISCUSSION

The extensive growth of the monokaryotic stage of *P. menthae* in the intercellular spaces of the leaf mesophyll and stem cortex, with a relatively low penetration of cells in these tissues (Fig. 1A) and the greater degree of invasion of the vascular system (Fig. 1B), was similar to that reported for infection of *Tussilago farfara* by *Puccinia poarum* (Al-Khesraji and Lösel, 1981; Larous and Lösel, 1993b). In contrast, the growth of dikaryotic intercellular hyphae and haustoria of the uredial stage was limited to the leaf mesophyll. The filamentous, monokaryotic haustorium appeared less specialized, lacked a neck-band and was not constricted at the point of entry to the host cell (Fig. 2A). The d-haustoria were more specialized, with a globular body and a long cylindrical neck, constricted at the penetration site (Fig. 2B), bearing a neck-band in its mid region (Fig. 4A). This was similar to other rust fungi cited above, and to the observation of Mims et al. (2001) on *Duchesnea indica* infected by the rust fungus *Frommeela mexicana* var. *indicae*, who found that the haustoria possessed a long slender neck with a neck-band and an expanded body that contained two nuclei. The haustorial mother-cell (HMC) wall of the d-haustorium was differentiated into four layers (Fig. 2C). The extra-haustorial matrix (EM) surrounding the haustorial body was bound by the invaginated host plasmalemma, forming the extra-haustorial membrane (EHM). Although Golgi bodies have not been recorded from rust fungi (Littlefield and Heath, 1979) and exist as single cisternae in most septate fungi, Golgi-like bodies were observed in this study, although infrequently, in d-haustoria of *P. menthae* (Fig. 2D) but not in m-haustoria.

The PA-TCH-SP staining procedures showed that polysaccharides are the major constituents of cell walls of *P. menthae*. All fungal walls reacted positively, but with differing intensities, those of the haustorial mother cell and haustoria of both monokaryotic and dikaryotic phases staining more densely than other parts of the fungus.

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**Fig. 1.** A. Light micrograph of stem cortex of mint (Cc). Note the dense growth of intercellular hyphae (Ie) of *P. menthae* monokaryon, contrasting with relatively infrequent penetration of these cells by the intracellular structures (Ia), and aecium base (ab). (Glut-OsO4-Toluidine blue). Bar =10 µm. B. Mint leaf showing an advanced stage of infection of the vascular system. The fungal structures (H) are within/and embedded in thickened wall of the xylem vessel (XV), and xylem parenchyma (XP). (Glut-OsO4-UA/PbC). Bar = 2 µm.
(Fig. 3A, B). As in the observations of Chong et al. (1981) on *P. coronata* in dikaryotic infections processed without osmium fixation, a method for extracting lipid from tissues (Hayat, 1970), PA-TCH-SP stainability was much reduced in the haustorial neck wall, mainly in the proximal region of the neck-band (Fig. 4A). The electron density of the neck wall was also considerably lower than in tissue conventionally fixed with glutaraldehyde and osmium (Fig. 4B). The PA-TCH-SP stainable material seen in conventionally fixed tissue (Fig. 3B) was much reduced in tissue not exposed to osmium fixation (Fig. 4B). This suggests the presence of lipopolysaccharides which are extracted by ethanol during dehydration.

The extra-haustorial matrix (EM), which had previously been suggested to be of host origin or an artifact of preparation (Littlfield and Heath, 1979), was shown to contain cellulose and glycoprotein by Chong et al. (1981, 1986), who claimed that the presence of cellulose may indicate that this component is of host origin. A similar conclusion was reached by Hickey and Coffey (1978) in their investigation of haustoria of *Melampsora lini*, another biotrophic pathogen.

![Fig. 2](image_url)

**Fig. 2.** A. Filamentous haustorium (H) in mesophyll cell. The fungal wall (FW) is densely stained and is of uniform thickness throughout the penetration region, there is no constriction at the penetration site, and the neck (HN) lacks a neck-band, nucleus (N), vacuole (V), vesicle (Vs). (Glut.-OsO₄- UA/PbC). Bar = 2 µm. B. D-haustorium of *P. menthae* infecting mesophyll cell (MC). The neck (HN) is constricted at the penetration site and the nuclei (N) are very regular in shape, haustorial mother cell (HMC), nucleolus (n). (Glut.-OsO₄-UA/PbC). Bar = 2 µm. C. Section through the haustorial mother cell (HMC) wall of a d-haustorium of *P. menthae* processed without osmium fixation. Note the four distinct layers in this region of the fungal wall, host cell wall (CW), haustorial neck (HN). (Glut.-UA/PbC). Bar = 0.25 µm. D. Haustorial mother cell (HMC) of d-haustorium penetrating mesophyll cell (MC). Note the Golgi-like structure within haustorial mother cell HMC (arrow), and the oval nucleus (N) containing a variable amount of eu- and heterochromatin. (Glut.-OsO₄-UA/PbC). Bar = 0.5 µm.
Evidence for the presence of cellulose in part of the EM of filamentous haustoria of *P. menthae* was provided by the electron-lucent appearance after cellulase treatment (Fig. 5A), a condition not observed in the EM of dikaryotic haustoria. The EM of both mono- and dikaryotic haustoria reacted positively to PA-TCH-SP staining, but the reaction was more intensive in the monokaryotic haustoria (Fig. 5B).

Treatments with protease resulted in a complete digestion of the EM in both phases of infection, while the invaginated host plasmalemma forming the EHM was ruptured in several points around the matrix (Fig. 6A). The positive reaction of the EM with PA-TCH-SP and its complete digestion by protease, provided evidence for the presence of glycoprotein. The EM of *Melampsora lini* was also found to contain protein but not cellulose (Coffey and Allen, 1983; Maticke et al., 1998). Although cellulose treatment digested host cell wall material, it did not affect the appearance of fungal walls of mono- or dikaryotic phases of infection, indicating an absence of cellulose.
from these structures, which is in agreement with Hu and Rijkenberg (1998) findings, who confirmed the absence of cellulose in the EM and host tubules associated with the invading haustorium of *P. recondita* f.sp. *tritici*.

In tissue subjected to protease treatment followed by PA-TCH-SP staining procedures, three regions could be distinguished in the haustorial neck of *P. menthae*, on the basis of wall composition (Fig. 6B). Region 1, near the point of fungal penetration was similar in chemical composition to region 3 distal to the neck-band, in that both of them had lost much of PA-TCH-SP stainable material, whereas region 2, proximal to the neck-band, but away from the penetration site reacted strongly with PA-TCH-SP. This indicates the presence of free polysaccharide in this region and of glycoprotein in the rest of the haustorial neck wall, as suggested by Chong et al. (1986) for *P. graminis*. The neck-ring appeared unaffected by any of these treatments. Similar differences in neck wall composition were reported in haustoria of *Albugo candida* (Coffey and Allen, 1983; Woods and Gay, 1983), in which the proximal haustorial neck wall was found to contain cellulose but the distal region was unaffected by cellulase and did not stain with PA-TCH-SP. However, in a similar study, Chong et al. (1981) found the entire haustorial neck wall of *P. coronata* to be uniform in composition.

This cytochemical study confirmed that d-haustoria of *P. menthae* are more specialized and complex in structure than the monokaryotic intracellular structures which resemble cytochemically the intercellular hyphae, even in an autoecious rust, where no host differences between mono- and dikaryotic development are involved.

The HMC wall was composed of four layers, and the haustorial wall of two layers which the above observations indicated to contain polysaccharide and protein. However, the haustorial neck wall differed greatly in chemical composition from the rest of the fungal walls and appeared to contain, in addition to polysaccharides, proteins and lipoproteins.

**Fig. 5.** A. Transverse section in mint mesophyll cell infected with the monokaryon haustorium (H) of *P. menthae* showing result of cellulase treatment. Note that the host cell wall (CW) and the extra-haustorial matrix (EM) have been almost completely removed, except for some stainable material remaining in the EM. (Glut.Cellulase-OsO4-UA/PbC). Bar = 0.25 µm. B. Transverse section of mature haustorium (H) of the monokaryotic stage of *P. menthae*. Note that the haustorium is surrounded by a thick layer of the extra-haustorial matrix (EM) which is less densely stained with PA-TCH-SP than the haustorial wall, the extra-haustorial membrane (EHM) is more densely stained than the membranes of the host cell. Note also the close association of the host mitochondria (M) and chloroplast (CH) with the haustorium (H). (Glut.-OsO4-PA-TCH-SP). Bar = 2 µm.

**Fig. 6.** A. Transverse section of d-haustorium (H) treated with protease then stained with PA-TCH-SP, the wall stained densely with PA-TCH-SP and appears unaffected by protease, while the extra-haustorial matrix (EM) is completely digested. Note the remaining portion of extra-haustorial membrane (EHM) (arrow), and a thin layer of fibrillar material surrounding the haustorial wall (arrow head) reacts strongly with PA-TCH-SP. (Glut.-protease-OsO4-PA-TCH-SP). Scale bar = 0.5 µm. B. L.S. neck of d-haustorial of *P. menthae*, after protease treatment. The neck wall of the haustorium (HN) shows a reduction of PA-TCH-SP staining compared with Fig. 3B due to digestion of some of the wall component. The remaining material is stained non-uniformly. The proximal portion next to the neck-band (region 2) is stained more densely than the other part of the neck (region 1 and 3). The neck-band is not stained (arrow heads). (Glut.-protease-OsO4-PA-TCH-SP). Bar = 0.25 µm.
Polysaccharides and proteins were found to be the major constituents of the EM of the mono- and dikaryotic haustoria of *P. menthae*, whereas cellulose was detected in the EM of monokaryotic haustoria only. The presence of cellulose in the EM supports the point of view that it is of host origin (Littlefield and Heath, 1979).

The reasons for and the significance of the differences in structure and functioning between mono- and dikaryotic phases of the life cycle of this autecious rust remain intriguing and unresolved questions. Since many of these features recur in this and other autecious species as well as in some heteroecious rusts, they are unlikely to reflect only physiological or structural differences in the host, but must relate to fundamental expressions of the mono- and dikaryotic genome of the pathogen. However, it was recently proven with the use of more sophisticated cytochemical methods and the analysis of haustorial function at the molecular level, that haustoria are involved in biosynthetic pathways, the suppression of host defenses, and in redirection or reprogramming the metabolic flow of the host (Mims et al., 2003; Mims and Richardson, 2004). The structural composition of this rust fungus seems to be similar to other previously studied rusts. However, more work has still to be done using more sophisticated cytochemical methods to give a better understanding of the host-pathogen interaction between *P. menthae* and its host plant.

**REFERENCES**


