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Histochemistry of papillae in potato tuber tissue infected with *Phytophthora infestans*

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Summary

Herbert Hächler and Hans R. Hohl. 1982. Histochemistry of papillae in potato tuber tissue infected with *Phytophthora infestans*. Botanica Helvetica *92:* 23-31. The main body of the papilla around the haustorium of *Phytophthora infestans* in potato tuber tissue contains two structural components, callose, (a β-1,3-glucan) and cellulose (a β-1,4-glucan). The evidence is based on a variety of staining and enzyme digestion studies: only a combination of β-1,3-glucanase and cellulase but neither enzyme alone (or other enzymes such as proteinase K, β-glucosidase, pectinase) are capable of digesting the papilla. The histochemical tests for lignin, pectin, cutin/suberin, protein and phenolic compounds were all negative. The extrahaustorial matrix stained positively with methylene blue and ruthenium red possibly due to the presence of pectin-like material or (glyco-)proteins.

Introduction

Haustoria of *Phytophthora infestans* in potato tissue of the resistant variety Eba are characteristically surrounded by an extrahaustorial matrix and by wall appositions (Hohl and Stössel 1976, Hohl and Suter 1976) in form of an encasement or papilla (Aist 1976). Papillae are more frequently found in the resistant Eba but have also been observed in the susceptible Bintje where predominantly collars are formed around the neck of the invading hypha. A quantitative study has shown that papillae in infected Eba outnumber those observed in Bintje by a factor of 20-30 per unit of host cell tissue colonized by the fungus (Hohl et al. 1980, Hächler and Hohl, in preparation).

The chemical composition of these papillae is not known. Staining of the papillae with the fluorochrome aniline blue (Hohl and Stössel 1976) indicates (Eschrich 1956) but not conclusively demonstrates the presence of callose (Faulkner et al. 1973, Smith and McCully 1978). Since papillae may represent a factor of resistance in this system (Hohl and Stössel 1976) it was essential to establish in more detail their chemical composition. In this study we present evidence that the two essential structural components of papillae are callose and cellulose. A preliminary report has been published elsewhere (Hohl et al. 1980).

Material and Methods

Phytophthora infestans (Mont.) de Bary, strain 515 (race 0) was used throughout. The fungus was grown in 9 cm plastic petri dishes on rye-dextrose agar (Crosier 1933) for 15 d at 15 °C. The sporangia were harvested with a few ml of sterile distilled water and incubated for 3 h at 4 °C to allow germination of zoospores. The suspension was then adjusted to give 4000 germ tubes/cm^2 of inoculated, freshly cut potato tuber surface.

Tubers of *Solanum tuberosum* L. cultivar Eba were obtained from the «Eidgenössische Forschungsanstalt für landwirtschaftlichen Pflanzenbau», Reckenholz, Zürich and were stored at 4 °C until used (less than six months total storage time). Eba possesses the resistance gene R_3 and has a high horizontal resistance.

Peeled tubers were superficially sterilized for 15 min in a 0.5 % sodiumtetraborate solution and then cut into 0.5 cm thick slices with a surface area of 1 cm^2. The slices were placed in a 9 cm plastic petri dish lined with moist filter paper. Following inoculation the specimes were kept at 16 °C and were tested after 48-72 h.

For histochemistry the material was fixed in a mixture af acetic acid conc. (5 %) – 40 % formaldehyde (10 %) – ethanol abs. (35 %) – water dist. (50 %) according to Gerlach (1977). Dehydration and embedding was either in acetone and polyethyleneglycol-methacrylate according to Ruthmann (1966), or in ethanol and paraplast according to Gerlach (1977).

Sections were cut on a Minot rotational microtome, 5-10 μm thick for methacrylate, 13-18 μm thick for paraplast embedded material, and mounted with an egg albumin-glycerine mixture.

For further treatment the embedding material was removed with 3 10 min treatments of xylol for paraplast and with 3 10 min treatments of 1-acetoxy-2-methoxy-aethane for methacrylate. Finally the sections were transferred to water via a series of ethanol/water concentrations.

To localize papillae the sections were prestained with an aqueous solution of 0.01 % aniline blue in M/15 phosphate buffer of pH 9.5 for 1 min, covered with a coverslip and observed in a Zeiss photomicroscope II with transmittance fluorescence attachment (exciter filter BG 12 and barrier filter comb. 53/44).

The position of the desired papilla was carefully sketched, the coverslip removed and the section thoroughly but carefully washed with water before subjecting it to further histochemical and enzymatic tests. Following these tests the coverslip was replaced and the papilla relocated on the basis of the sketch. The results of the treatment were photographically recorded on Agfachrome professional type 50 L.

The following histochemical tests were performed:

(1) Lacmoid for callose (Jensen 1962),
(2) Ponceau S for callose (Ulrychová et al. 1976),
(3) calcofluor for cellulose (Rohringer et al. 1977),
(4) iodine-potassium iodine (I_2Kl-H_2SO_4) and iodine zinc chloride (I_2ZnCl_2) for cellulose (Jensen 1962),
(5) methylene blue for pectin-like substances (Gerlach 1977),
(6) ruthenium red for pectin-like substances (Jensen 1962),
(7) sudan IV for cutin and suberin (Sherwood and Vance 1976),
(8) acridine organe for lignin/phenolic (also RNA) compounds (Verbeek 1977),
(9) toluidine blue 0 for phenolic compounds (Ling-Lee et al. 1977, Petersen et al. 1978),
(10) phloroglucin-HCl (Johansen 1940), Mäule-reaction (Frohne 1974) for lignin.

Enzyme digestion assays: The following enzymes were used: endo-β-1,3-glucanase QM 1032 from *Rhizopus arrhizus* (obtained from Dr. Reese, US Army Natick Development Center, Mass. USA), cellulase (Sigma Corp., USA) pectinase (Sigma Corp.), β-glucosidase from almonds (Fluka, Buchs, Switzerland). These enzymes were used at 5 mg/ml in 0.2 M acetate buffer at pH 5.5. Proteinase K (from fungi, Merck) was used in 0.1 M tris-HCl buffer at pH 7.5. Incubation with all enzymes was at 28 °C for 2-24 h as indicated in the text.

Control experiments showed that cellulose and pectin were removed from fixed host tissue wall as indicated by the negative reaction of the calcofluor and ruthenium red test respectively after but

not before enzyme treatment. This demonstrated that enzymatic digestion of these compounds was still possible following fixation and thorough washing of the material. Cellulase, pectinase and β-1,3-glucanase were tested for the presence of contaminating enzymes: no β-glucosidase activity was measured but cellulase contained traces of β-glucosidase, therefore, β-glucosidase was tested individually and in combination with cellulase during the experiments. No cellulase activity was measured in the pectinase and the β-1,3-glucanase. Cellulase but not pectinase contained measurable amounts (22% of the β-1,3-glucanase activity) of β-1,3-glucanase activity as determined by release of glucose from laminarin.

Results

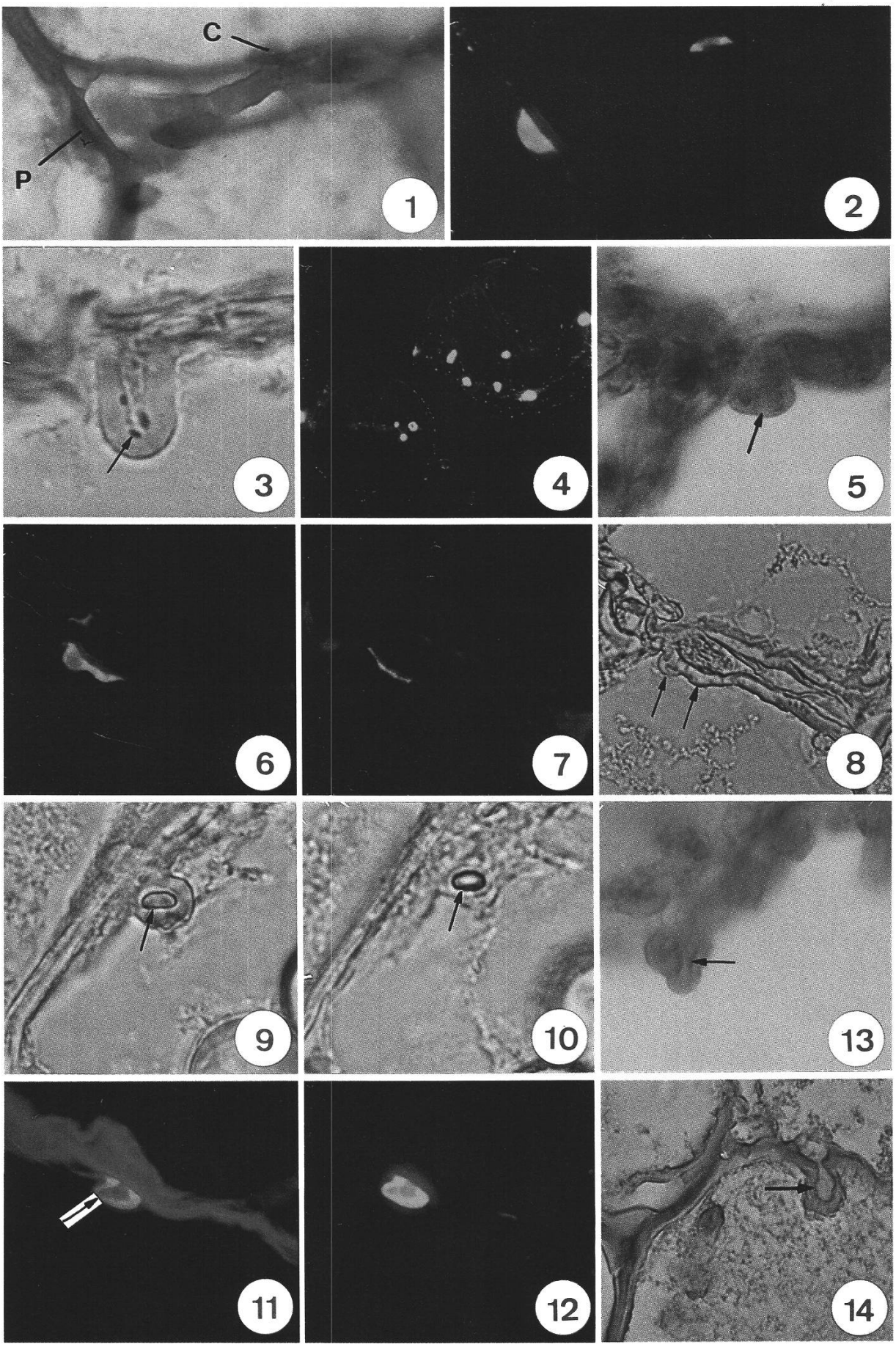
Wall appositions occur mainly in form of papillae (Fig. 2, 3, 9, 11, 12) or collars (Fig. 2, 4). Size and form of papillae may vary considerably. Most frequent are more or less hemispherical structures (Fig. 2, 9) with diameters of 5-10μm. Others are cylindrical (Fig. 3), branched or very irregular and up to 30 μm large. Whereas in the uppermost cell layers of the infected tuber tissue small and slender papillae predominate, bulbous, larger forms are more typical for the deeper penetration sites.

Callose: Papillae have no autofluorescence. After staining with aniline blue they show a very strong yellow fluorescence (Fig. 1, 2, 4, 6). A much weaker and more greenish fluorescence is also shown by the starch and the cell walls. In bright field the papilla appears blue with aniline blue provided the pH is lowered to 5.5 with a buffer solution. An additional positive reaction for callose in form of a faint blue staining was obtained with lacmoid (Fig. 5), whereas Ponceau S gave ambiguous results. The digestion experiment with β-1,3-glucanase provided the following result: after 3 h in the enzyme solution the renewed aniline blue test was negative (Fig. 6, 7). However, the microscopic appearance of the papilla remained unchanged by this treatment (Fig. 8).

Cellulose: A positive reaction for cellulose was obtained with calcofluor (Fig. 11, 12). The yellowish green (Fig. 11) instead of the pure green fluorescence might be due to the presence of callose known to fluoresce yellow with calcofluor (Hughes 1975). The presence of cellulose could be verified with the digestion tests: a 12 h treatment with cellulase reduced the size of the papilla by about 20%. However, if cellulase and β-1,3-glucanase were applied simultaneously the entire papilla was removed after 2 h (Fig. 9, 10). It should be kept in mind that β-1,3-glucanase alone had no effect on the structural integrity of the papilla; neither did (1) β-glucosidase, (2) a combined treatment of cellulase and pectinase or (3) of β-1,3-glucanase and pectinase, even after incubation of up to 24 h. The classical tests for cellulose with iodine-potassium iodine and iodine-zinc chloride were negative, most likely due to steric hindrance by the closely associated callose (see discussion) present in the papilla.

Lignin: The tests for lignin (phloroglucine-HCl, Mäule-reaction and acridine orange) were all negative indicating that there are no lignins in the papillae or only in amounts below the sensitivity of the tests.

Pectin: Both ruthenium red and methylene blue did not or only weakly stain the main body of the papilla i.e. the wall apposition material. However, the thin extrahaustorial matrix (see Hohl and Stössel 1976) adjoining the penetration hypha stains intensively with both stains (Fig. 13, 14). Pectinase even after 12 h had no visible effect on the structure and stainability of the papilla. Possibly the extrahaustorial matrix does not contain pectin but glycoproteins which could give similar staining reactions.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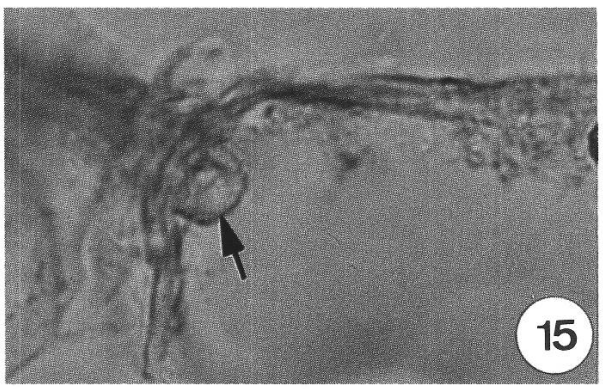

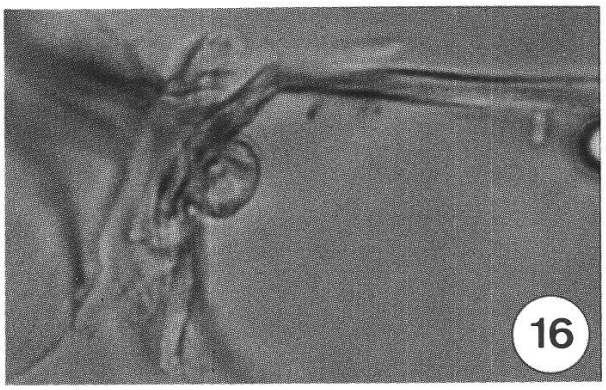

Figure Legends

Fig. 1 and 2. Tissue of the resistant Eba, 60 h following infection, × 800. Papilla (P) and collar (C) are difficult to trace in the toluidine blue stained section (Fig. 1) but clearly distinguishable when viewed in the fluorescence microscope after aniline blue staining (Fig. 2).

Fig. 3. Well developed papilla in the resistant Eba surrounding an invading fungal hypha. The papilla contains three darkly staining granula (arrow) not uncommonly encountered in these structures. Unstained section, 72 h post infection, × 1300.

Fig. 4. Two isolated cells scraped from infected tuber tissue showing multiple perforation by the parasite as evidenced by the yellow fluorescing collars and papillae. Aniline blue staining, × 150.

Fig. 5. Staining of papilla for callose with lacmoid. The bluish tint (arrow) is a positive indication for the presence of callose, × 950.

Fig. 6-8. Effect of β-1,3-glucanase on aniline blue staining and structure of papillae, × 600. – Fig. 6. Heavy staining of the papillae before enzyme treatment. – Fig. 7. The same papillae after a 3 h treatment with β-1,3-glucanase and restained for 1 h with aniline blue: the papillae have lost their affinity for this dye. – Fig. 8. The structure of the papillae (arrow) has remained virtually unchanged by the enzyme treatment.

Fig. 9 and 10. Enzyme digestion of papillae with a combination of β-1,3-glucanase and cellulase. Sections unstained, × 950. – Fig. 9. Papilla before, – Fig. 10, papilla following a 2 h combined enzyme treatment. Only the centrally located haustorium (arrow) remains while the entire papillar body has disintegrated.

Fig. 11 and 12. Test for cellulose with calcofluor. – In Fig. 11 the papilla has been stained with calcofluor. The greenish color (arrow) is a positive indication for the presence of cellulose. The yellowish tint is probably due to the presence of callose known to react in this manner with calcofluor. – Fig. 12. The same papilla poststained with aniline blue resulting in a strong yellow coloration of the papilla. Notice absence of aniline blue staining in the cell wall. Both figures × 600.

Fig. 13 and 14. Papilla stained with ruthenium red (Fig. 13) and with methylene blue (Fig. 14) for pectin-like substances. Only the extrahaustorial matrix (arrow) reacts positively. Fig. 13, × 950; Fig. 14, × 600.

Fig. 15 and 16. Enzyme digestion test with proteinase K. – Fig. 15. Papilla (arrow) before enzyme treatment. – Fig. 16. Papilla following a 24 h treatment with proteinase. Notice that the cytoplasm along the cell wall and around the papilla has been cleared away, yet the structure of the papillar body itself remains unchanged. Both figures × 950.

Cutin and suberin: There was no indication for the presence of cutin or suberin based on the Sudan IV staining procedure indicating that these substances are not part of the papilla or present only in minor quantities.

Protein: The presence of protein as «contaminant» of papillae is indicated by the observation of Hohl and Stössel (1976) who observed numerous small pockets of cytoplasmic material entrapped in the main body of the papilla. To test whether or not protein was present as a structural part of the papilla digestion experiments with proteinase K were carried out. Even after 24 h there was no indication of structural changes taking place as a result of the enzyme treatment, whereas the cytoplasm present around the papillae and along the cell walls had been completely removed (Fig. 15, 16).

Phenolic compounds: Neither toluidine blue nor acridine orange which stains walls of wounded or infected potato tissue thought to contain phenolic compounds (Hächler and Hohl, in preparation) brought about staining of the papilla, indicating absence of phenolic compounds in this structure.

Discussion

Papillae induced by *Phytophthora* spp. and the obligate members of the *Peronosporales* are typically composed of an inner, electron dense extrahaustorial matrix (ema) and a thick, outer and electron transparent layer of material termed wall appositions (Bracker and Littlefield, 1973). Whereas in some cases the wall appositions appear as a more or less uniform layer (e.g. Hohl and Suter 1976), in others a more electron dense outer layer may be distinguished from an inner one bordering the extrahaustorial matrix (Chou 1970, Kajiwara 1973, Kröber et al. 1979, Wehtje et al. 1979). Little is known of the chemical make-up of these papillae. Based on staining reactions (mainly with aniline blue) the presence of callose has been reported for a variety of papillae (Aist 1976) and seems to be the most widely distributed component so far investigated. It has also been frequently reported to be present in oomycete-induced papillae (Fraymouth 1956, Davison 1968, Sargent et al. 1973, Hohl and Stössel 1976, Wilson and Coffey 1980). However, with exceptions (Wilson and Coffey 1980) no adequate identification of either callose or cellulose (Aist 1976) as constituents of wall appositions has so far been presented.

A main result of this study is the positive identification of callose and cellulose as structural elements of wall appositions in this system. Since our evidence is based mostly on histochemical evidence, some caution is appropriate in interpreting the results. This also applies to the negative reactions obtained for pectin, lignin, cutin, suberin and phenolics. According to several authors (Faulkner et al. 1973, Smith and McCully 1978) aniline blue is not a stain specific for callose. Following the arguments of Smith and McCully (1978) the specific locations of the intense aniline blue induced fluorescence does indicate some differential feature of the wall architecture. This localized difference might possibly be chemical in terms of the types of compounds and polysaccharides present at the aniline blue positive sites, but it might as well be physical in terms of the arrangement or stacking of the polysaccharides within this area. The removal of aniline blue induced fluorescence by a β-1,3-glucanase as observed in our study and that of Wilson and Coffey (1980) represents direct evidence for the presence of a compound possessing β-1,3-glycosidic linkages. Our observation that the structure of the papilla is

not substantially altered by this enzyme is a positive indication that callose is not the only structural component present.

The presence of cellulose in wall appositions is indicated by the positive staining reaction with calcofluor. Yet this substance has also been shown to have affinities for other β-linked polymers (Hughes and McCully 1975). Here, too, the enzyme digestion experiments provide superior evidence for the presence of this polysaccharide. The fact that the main body of the papilla is removed by the combined action of β-1,3- and β-1,4-glucanases but not by either enzyme alone strongly favors the view that β-1,3- and β-1,4-linked polymers are responsible for the structural integrity of papillae and probably of other wall appositions such as collars or sheet-like structures sometimes covering the inside of infected potato cell walls (Hächler and Hohl, in preparation). It should be kept in mind though that there might be several chemically different kinds of «callosities» (Eschrich 1956) and it is not known whether a given host always produces the same kind of «stress-callose» upon stimulation by different challenges such as mechanical or chemical injury or infection by viruses, bacteria or fungi.

The results obtained so far suggest that papilla formation in our system constitutes a process related to wall formation. Since the presence of both callose and cellulose material has been indicated in papillae induced by other groups of fungi in a variety of hosts (e.g. Sherwood and Vance 1976) this might represent a rather general phenomenon. It appears that a signal from the invading parasite induces the host cell to hastily build up a layer of wall-like material in a locally confined area. The view that papillae in many cases are indeed «wall-like» has been postulated long before ultrastructural or histochemical evidence became available (see Aist 1976). It is further strengthened by the observation that callose takes part in normal, early cell wall formation (Klein et al. 1981, Waterkeyn 1981) and may even be a precursor of cellulose (Meier et al. 1981). Papillae may also «differentiate» by e.g. lignification as do secondary cell walls (Aist 1976, Sherwood and Vance 1976, Ride and Pearce 1979, Zeyen and Bushnell 1979). The basic relatedness between cell wall and papilla is furthermore convincingly demonstrated by the observation of Manocha and Letourneau (1978) that papillae of a mycoparasitized fungus with chitinous walls also contain chitin, i.e. are chemically related to the host cell wall.

Despite these common properties the two structures also certainly differ in chemical composition and architecture as indicated by (1) differential staining with e.g. aniline blue, (2) differential reaction toward enzyme treatment, the papillae being more resistant to enzyme attack, as also noted for wheat papillae by Ride and Pearce (1979), and (3) the inclusion of cytoplasmic debris in papillae but not in regular walls.

Regarding the extrahaustorial matrix (ema) our investigation, being limited by the resolving power of the light microscope, does not add substantially to the knowledge of its composition. The positive reaction obtained with methylene blue and ruthenium red, both rather unspecific stains for pectin (Luft 1976) that also react positively with other polysaccharides and (glyco-)proteins, is compatible with the result of Hickey and Coffey (1978) who localized cellulose and (glyco-)proteins in the ema of the *Peronospora pisi/ Pisum sativum* system. The host-arbuscule matrix in endomycorrhiza also shows properties resembling those of ema, i.e. positive ruthenium red staining and fibrillar (cellulose) elements (Scannerini and Bonfante-Fasola 1979).

Since the ema constitutes the interface between the fungal wall and the host-derived wall apposition a more detailed investigation of its composition and nature is likely to yield considerable information on the processes governing these host-parasite interactions.

Zusammenfassung

Die vom resistenten Wirt *(Solanum tuberosum)* gebildete und das Haustorium des eindringenden Parasiten *(Phytophthora infestans)* umschließende Papille besteht aus zwei Strukturkomponenten, nämlich Cellulose (ein β-1,4-Glucan) und Kallose (ein β-1,3-Glucan). Die Resultate stützen sich auf eine Reihe von histochemischen Tests und Enzymabbauversuchen: Die positive Färbung mit Anilinblau und Lacmoid deutet auf die Anwesenheit von Kallose, die Färbung mit Calcofluor auf Cellulose. Nur eine kombinierte Anwendung von β-1,3-Glucanase und Cellulase, nicht aber die der Einzelenzyme, führte zum Abbau des Kapselmaterials. Die Behandlung mit Proteinase K, β-Glucosidase oder Pektinase blieb ohne sichtbaren Einfluß. Die histochemischen Tests für Lignin, Pektin, Cutin, Suberin, Protein und phenolische Verbindungen verliefen ebenfalls alle negativ. Die extrahaustoriale Matrix, eine dünne Zwischenschicht zwischen Haustorium und Papillenkörper, ließ sich deutlich mit Methylenblau und Rutheniumrot anfärben, ein Hinweis für das Vorhandensein von pektinähnlichem Material oder von (Glyco)-Proteinen.

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