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THE POLLEN-STIGMA INTERACTION IN THE GRASSES. I. FINE-STRUCTURE AND CYTOCHEMISTRY OF THE STIGMAS OF HORDEUM AND SECALE

J. HESLOP-HARRISON and Y. HESLOP-HARRISON

Welsh Plant Breeding Station. Plas Gogerddan, nr Aberystwyth SY23 3EB, United Kingdom

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SUMMARY

The papillar cells of the stigmas of *Secale cereale* and *Hordeum bulbosum* show characteristics suggesting specialisation for both external and internal secretion. Each papilla terminates in a reflexed pollen-receptive tip bearing a proteinaceous surface pellicle overlying a thin mucilaginous layer, which in turn adjoins the markedly discontinuous cuticle. The basal part of each papilla contributes to the axis of a secondary stigma branch. A distinct layer of the papilla wall underlaying the cuticle is continuous with interstitial material in the axis of the branch. This material, which forms the pollen-tube transmitting medium, is constituted principally of acidic pectic polysaccharides, but contains a protein component, seemingly an internal secretory product of the basal parts of the branch cells. The papillar cells are bounded by a further distinctive wall layer. This is PAS reactive, stains with phosphotungstic acid-HCl, and also with the fluorescent polysaccharide stain, calcofluor white, which has been considered to have affinity with 1,4-linked glucans. The layer shows no evidence of a microfibrillar structure, however, and is not birefringent.

Dictyosomes are present during the final maturation of the stigma papillae, but they show no special evidence of secretory activity. On the other hand, paramural bodies are frequent in the papillar cells, and in the branch axes they are usually associated with vesicle swarms in the adjacent intercellular spaces. It seems likely that the paramural bodies form a granulocrine secretory system producing both the surface secretions of the papillae and the protein-polysaccharide constituents of the intercellular spaces in the branch axes.

The paper is the first of two; the second will be concerned with the responses of the stigma to pollination.

1. INTRODUCTION

The grass stigma responds to compatible and certain types of incompatible pollination by a series of reactions which lead ultimately to the death of the affected cells in the receptive and pollen-tube transmitting tissues (KATO & WATANABE 1957). In the initial stages of the response, observable very soon after the first contact of an emerging pollen tube tip with the stigma surface, the permeability of the contiguous stigma papillae increases, and the plasmodesmata connecting them with adjacent cells become occluded with callose. The papillae are thus disabled, and left incapable of receiving further pollen. We have argued that whatever other significance these responses may have they must operate to control pollen germination on the stigma, and so to reduce pollen-

tube competition in the transmitting tracts and ovary (J. HESLOP-HARRISON 1980). They are, accordingly, of considerable importance in regulating reproductive behaviour. Since the original publication of KATO & WATANABE (1957), there have been few references in the literature to the stigma responses in the grasses, and no detailed observations on the phenomena themselves using modern methods. In the present paper we provide an account of some of the ultrastructural and cytochemical features of the stigmas of *Hordeum* and *Secale*, and in the second we will report on an investigation of the details of the stigma response to pollination in these and other grass genera.

2. MATERIALS AND METHODS

The principal observations were made on *Secale cereale* L. cv. Rheidol and *Hordeum bulbosum* L., grown in field plots or with artificial illumination under glass. Young inflorescences were removed as required and brought into the laboratory, where anthesis was promoted by exposure to infra-red lamps.

Neutral red (R.A.Lamb, 1% in 1% acetic acid) and the fluorochrome acridine orange (Allied Chemicals, 0.1% aqueous) were used to map dye penetration. Fresh, intact stigmas were exposed to the dye for 2–3 min, and then rinsed quickly and mounted in dilute glycerol for observation. For the localisation of surface secretions, fresh stigmas were stained with ruthenium red (TAAB; 0.02% aqueous) or toluidine blue (R.A.Lamb; 0.01% in 0.1 M acetate buffer at pH 6.0). The stigmas were immersed in the stains until satisfactory differentiation was obtained, and then rinsed and mounted in dilute glycerol. Non-specific esterase was localised in intact stigmas using α-naphthyl acetate as a substrate and tetrazotised o-anisidine as a coupler (PEARSE 1972). The reaction was allowed to proceed 2–3 min, and the stigmas were then washed thoroughly and mounted for observation.

Material for electron microscopy was fixed in 1.5% glutaraldehyde in 0.05M phosphate buffer at pH 7.2 containing 8% sucrose for 1–4 h at room temperature, and post-fixed in 1% osmium tetroxide in the same buffer for 1.5–2 h at c. 4 C. The samples were then washed in buffer, transferred to water, dehydrated through an ethanol series and passed through propylene oxide before embedding in Araldite. Thin sections were cut with diamond knives, and post-stained with uranyl acetate and lead citrate for the observation of general ultrastructural features. Sections were also post-stained with phosphotungstic acid (1% in 10% HCl). This procedure has been said to stain the plasmalemma specifically (ROLAND 1979), but we find that it is mainly effective in giving electron density to components of the wall that stain with alcian blue and ruthenium red, suggesting an affinity with acid pectic polysaccharides (Y. & J. HESLOP-HARRISON 1980; see also PEASE 1966). For the ultrastructural localisation of polysaccharides with vicinal glycol groups, the Thiery procedure was used (THIERY 1967). Sections were collected on gold grids, and oxidised for 25 min at room temperature in 1% periodic acid. They were then washed and exposed overnight to 0.2% thiosemicarbazide in 20% acetic acid, taken through an acetic acid series to water, and

immersed in 1% aqueous silver proteinate in the dark for 20–30 min before washing and drying for observation.

Material for optical microscopy was fixed in 1.5% glutaraldehyde as for electron microscopy, and embedded in JB4 resin (Polysciences Inc.) without osmication. Semi-thin sections (1.0–1.5 μm) were cut with glass knives. The staining procedures used were (a) the PAS method (PEARSE 1972) for the general localisation of polysaccharides with vicinal glycol groups; (b) calcofluor white (Polysciences; 0.001% aqueous) for 1,4-linked glucans (MAEDA & ISHIDA 1976); (c) alcian blue 8GX (R.A. Lamb, 1% in 3% acetic acid) and ruthenium red, applied as above, for pectic polysaccharides; (d) decolourised aniline blue (BDH, 0.05% at pH 11) for the localisation of callose (ESCHRICH & CURRIER 1964); (e) auramine O (Gurr, 0.01% in 0.05 Tris-HCl buffer, pH 7.2) for cutin (Y. HESLOP-HARRISON 1977), (f) scarlet R (saturated in 70% ethanol) for lipids (GURR 1965), and (g) Nobel stain (Coomassie blue R250, azocarmine and amido black all at 0.25% in water: ethanol: acetic acid, 60:30:10, v/v) for protein. Resin-embedded sections were also stained with the metachromatic stain toluidine blue, applied as above.

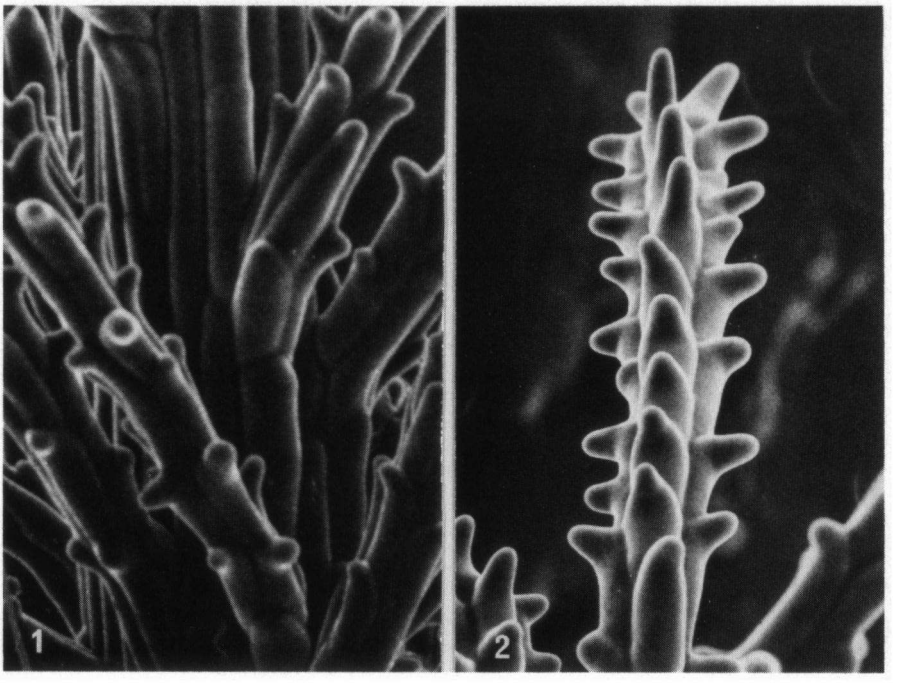
3. OBSERVATIONS

3.1. Stigma structure

The general morphology of the stigma of *Secale cereale* (rye) may be grasped from the scanning electron micrographs of *figs. 1* and *2*. As is characteristic in most Gramineae, the stigma is twin-branched and feathery, and of the dry, papillate type (Y. HESLOP-HARRISON & SHIVANNA 1977). The secondary branches arising from the two principal axes are 4 (rarely 5) cells in section at the base, passing through a stretch of 4 cells in section to terminate in a single cell. A vascular strand traverses the core of the main axes, but the branches are without vasculation.

The papillae are unicellular, with reflexed tips. The principal fine-structural features of the mature, receptive papillae are seen in the electron micrographs of *figs. 3* to *6*. The nucleus occupies a central position in the cell, which is highly vacuolate. The cells possess the usual complement of organelles. Mitochondria have moderately well-developed cristae, and the plastids, which have few lamellae, only occasionally contain starch. In the mature papillae dictyosomes are relatively few in number, and have an 'inactive' configuration, with few peripheral vesicles. Profiles of ribosomal endoplasmic reticulum occur throughout the cytoplasm, and also numerous free ribosomes.

Tonoplasts often appear to be absent from the papillae of exserted stigmas following the usual fixation and post-staining procedures. This is true even for cells in which the plasmalemma and other membranes are well defined (*fig. 22*), suggesting that the vacuolar membranes may be absent from the mature receptive papillae. The vacuoles themselves are often – but not invariably – filled with a weft of fibrils with electron-staining properties suggestive of a proteinaceous character (*fig. 6*).



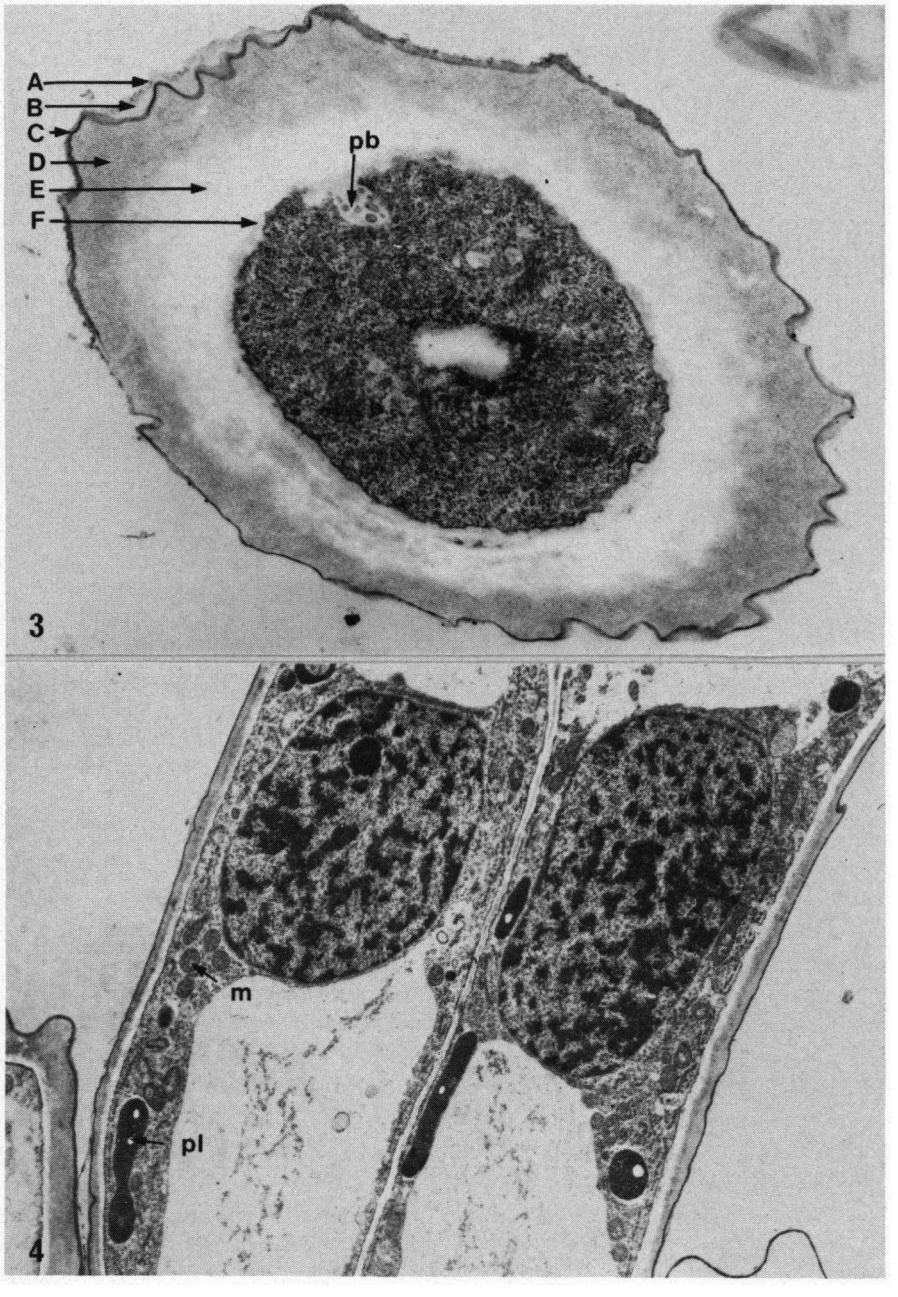
Figs. 1 & 2. Scanning electron micrographs of stigmas of *Secale cereale*, fresh uncoated preparations.
Fig. 1. Axis of the primary stigma branch, showing the insertion of the secondary branches. × c. 375.
Fig. 2. Secondary stigma branch. The branch is composed of the basal parts of the papillae, which are mostly in four, or very occasionally five, ranks. × c. 400.

Paramural bodies occur frequently in the papillar cells (*figs. 3* and *6*). The significance of these is considered in a later section in connection with the stigma secretions.

3.2. Wall stratification

All external faces of the papillar cells are cuticularised. In the basal zone, the cuticle has a thickness of 190–250 nm in rye, and has ocasional irregular discontinuities. In the region of the reflexed tip these discontinuities are much more frequent. The stratification of the wall in this distal zone, the pollen-receptive part of the papilla, is illustrated in the diagram of *fig. 7*, which may be compared

Figs. 3 & 4. Electron micrographs of the stigma of *Secale cereale*, standard uranyl acetate-lead citrate post-staining.
Fig. 3. Slightly oblique transverse section near the tip of a papilla. The wall shows the layers A–F of the diagram in *fig. 7*. The tip zone is usually densely filled with cytoplasm, with numerous ribosomes. A paramural body (pb) is visible. × c. 15,000.
Fig. 4. Longitudinal section of a secondary branch, showing the basal regions of two papillae; m, mitochondria; pl, plastid. × c. 4,750.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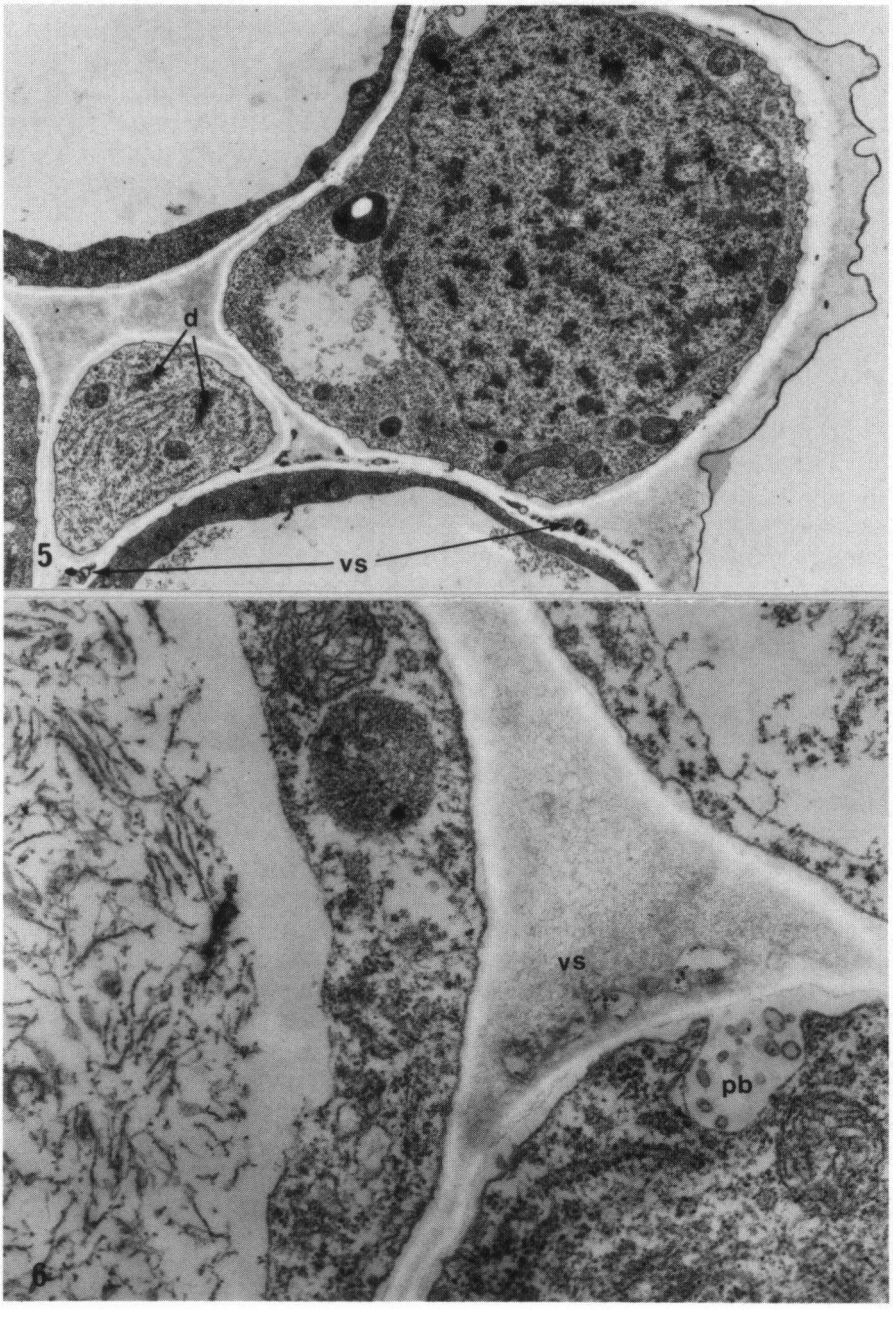
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with the electron micrographs of *figs. 3–6, 8, 9* and *20–22*. The staining reactions of the wall and its coatings are listed in *table 1*.

The papilla bears an outer coating (A) some 15–20 nm in thickness (*figs. 20–22*), corresponding to the pellicle found in other dry-stigma families (MATTSSON et al. 1974; HESLOP-HARRISON, KNOX, HESLOP-HARRISON & MATTSSON 1975). The coating is separated from the surface of the cuticle by an intervening layer (B) of intermediate electron density with standard post-staining procedures. This layer has a thickness of 100–110 nm over most of the surface of the papilla, widening over crevices and where the papilla unites with other cells at the base of the reflexed tip (*figs. 21* and *22*).

The discontinuous cuticle (C) is illustrated in *figs. 20–22*. The electron micrographs show that the discontinuities are bridged both by the overlying pellicle and the layer B. The stratum of the wall underlying the cuticle (D) has a thinly dispersed microfibrillar content (*figs. 20* and *21*), and the staining responses indicate a considerable pectic component. This layer adjoins a distinct stratum (E) which is invariably highly electron transparent following uranyl acetate and lead citrate staining (e.g., *figs. 3* and *5*). Electron density is given by PTA staining (*fig. 8*). The layer is PAS reactive, and stains heavily with the fluorochrome, calcofluor white (*fig. 10*). A further stratum (F) of varying thickness and intermediate electron density is usually evident between the layer D and the plasmalemma.

The reactivity of the wall in the Thiery procedure is illustrated in *fig. 9*. The densest silver deposits are over the layer E, electron transparent following standard uranyl acetate-lead citrate staining. The plasmalemma is also heavily labelled, but there is little reactivity associated with the tenuously microfibrillar layer D, which also has low PAS stainability. The bases of the stigma papillae unite to form the secondary stigma branches (*fig. 2*), and in this region the outer walls show a similar stratification (*figs. 5* and *22*). The inner walls naturally lack the cuticle and the overlying layers A and B of *fig. 3*, but the layers D, E and F are present. The material of stratum D is seen to be continuous with that of the layer separating the cells and occupying the enlarged intercellular spaces in the core of the branch (*figs. 5* and *6*); morphologically, therefore, it corresponds to the middle lamella. The intercellular spaces and surface clefts are readily resolved with the optical microscope, permitting some of the cytochemical properties of the interstitial material to be established. Most notable are the affinity for protein stains (*fig. 12*) and the presence of weak esterase activity.

Figs. 5 & 6. Electron micrographs of the stigma of *Secale cereale*, standard uranyl acetate-lead citrate post-staining.

Fig. 5. Transverse section at the junction point of a papilla with a secondary branch. The continuity between wall layer D and the interstitial material of the branch axis which forms the pollen-tube transmitting tract is seen, and also the highly electron-transparent wall layer, E. vs, vesicle swarms in the intercellular spaces; d, dictyosomes. × c. 15,000.

Fig. 6. Detail of the interstitial material in a branch axis. A paramural body is seen at pb, and a vesicle swarm (vs) in the intercellular space roughly in register with it. The vacuole of the cell on the left contains numerous thick fibrils, probably proteinaceous. × c. 35,000.

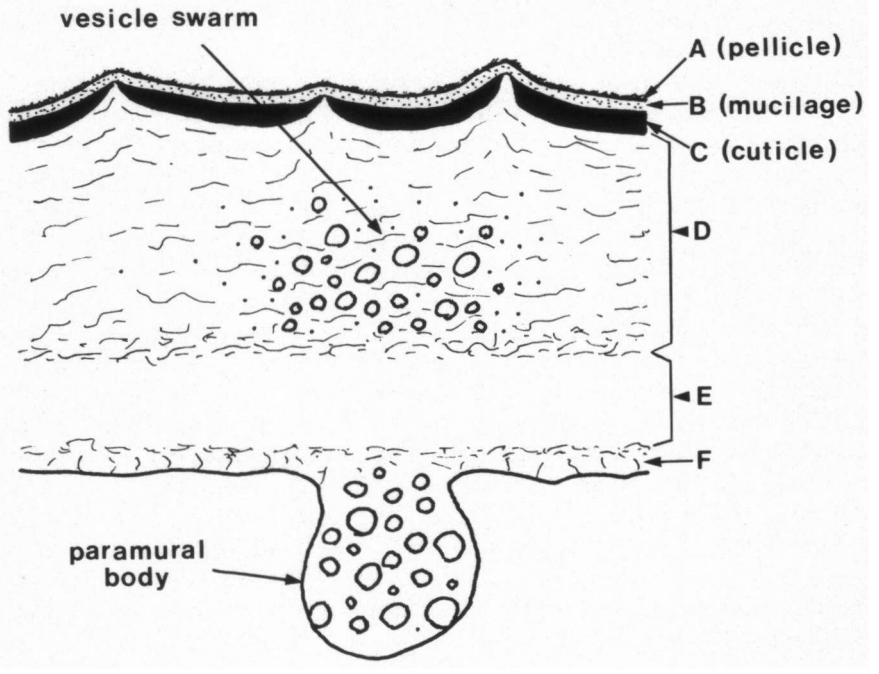


Fig. 7. Diagram of the structure of the papilla wall, showing the six layers, A–F, evident in electron micrographs. Further explanation in the text.

Table 1: Staining reactions of the wall strata of the stigma papillae of *Secale cereale* and *Hordeum bulbosum*, observed in JB4-resin embedded material sectioned at 1–1.5 μm. Layers identified as in *fig. 7*, apart from layers A and F which cannot be resolved with the optical microscope.

	Wall layers			
	B	C	D	E
PAS	O	O	+	+++
Calcofluor white	O	O	O	+++
Toluidine blue	+ (pink)	O	+++ (pink/purple)	+++ (blue)
Alcian blue	+	O	+++	++
Ruthenium red	+	O	++	++
Aniline blue, pH 11	O	O	O	O
Auramine O	O	+	O	O
Scarlet R	O	(+)	O	O
Nobel stain	O	O	++	O

Key to symbols: +++, strong staining reaction; ++, moderate or localised staining; +, light staining only; (+) staining response ambiguous. No fluorescence with decolourised aniline blue was observed in unpollinated, undamaged stigmas, indicating the absence of callose.

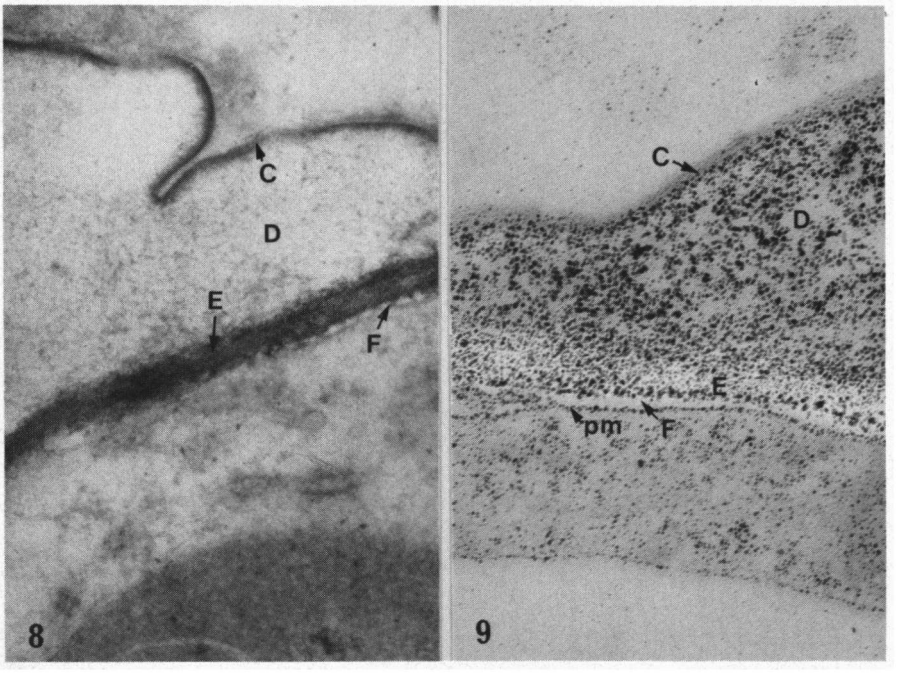
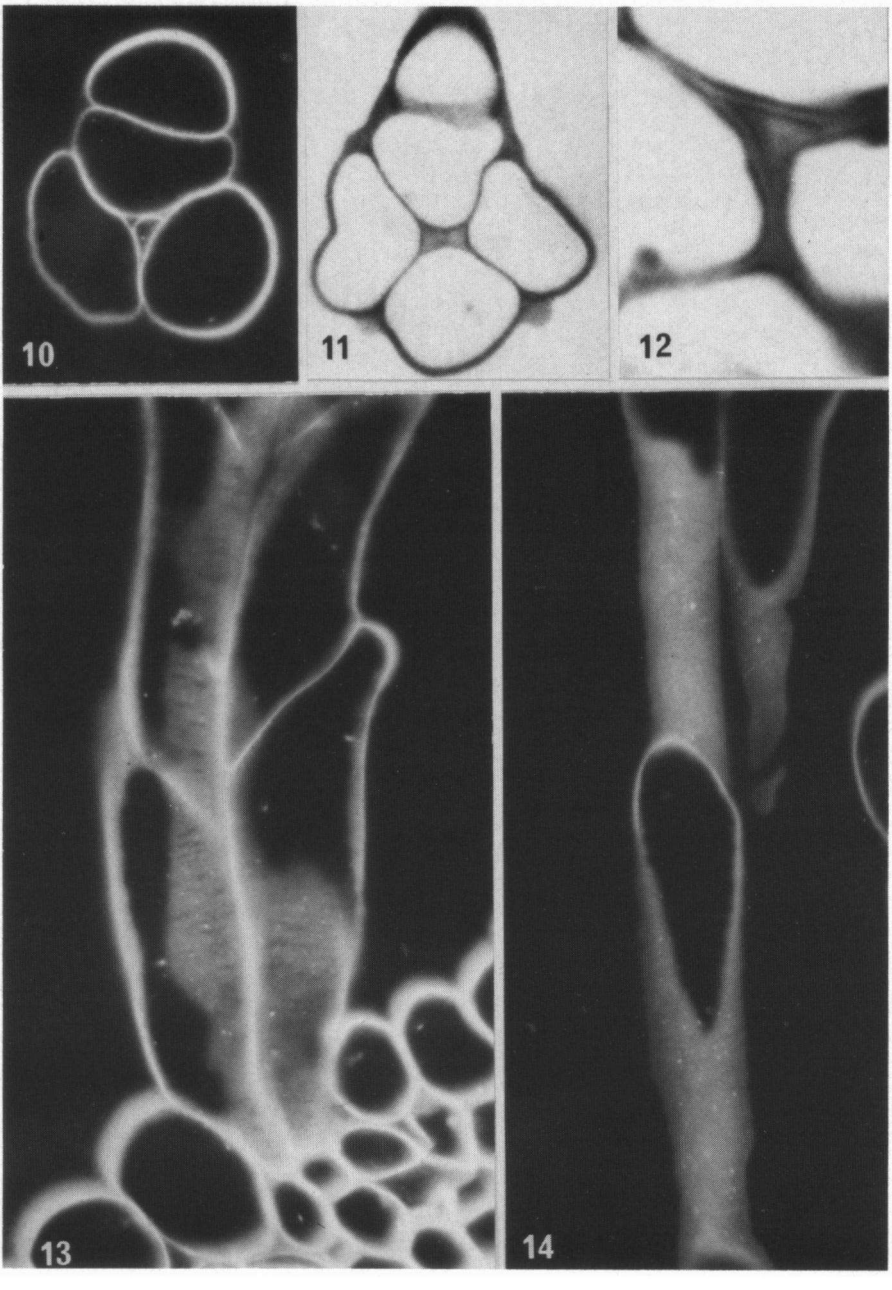


Fig. 8. Electron micrograph of the wall of a papilla of *Secale cereale*, PTA-HCl post-staining, labelling of wall layers as in Fig. 7. With this treatment, the wall layer E, electron transparent with standard uranyl acetate-lead citrate staining, acquires high electron density. × c. 70,000.
Fig. 9. Electron micrograph of the wall of a papilla, Thiery procedure. Wall layers labelled as in *fig. 7*; pm, plasmalemma. × c. 75,000.

3.3. Secretions and secretory systems

Brief toluidine blue and ruthenium red staining reveals the presence of surface secretions on the papillae, as dispersed droplets over the receptive tips (*fig. 16*), or as more considerable accumulations in the basal crevices (*fig. 15*). The material is a viscous fluid, not readily dispersed in water, and the staining properties are suggestive of a mucopolysaccharide. It is probable that it is this that forms the layer B visible in electron micrographs (compare, e.g., *fig. 22*).

A Golgi system is present in the cells of the papillae, but the dictyosomes are relatively inactive during the period when the interstitial material of layer D – itself of the nature of an internal secretion – and the extra-cuticular surface secretions are accumulating, at least so far as a judgment can be made from electron microscopic evidence. On the other hand, paramural bodies are present abundantly in all the papillae, notably near the cell junctions. Examples are illustrated in *figs. 3* and *6*. The bodies are frequently found in register with vesicle swarms in the intercellular spaces (*fig. 6*). The vesicle accumulations in the wall layer D in the surface clefts are often very extensive (*fig. 18*), and possibly contribute to the protein staining observable with the optical microscope in these regions.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Like other stigmas of the 'dry' type, that of the grasses shows surface esterase activity, associated with the pellicle layer A (HESLOP-HARRISON, HESLOP-HARRISON & BARBER 1975). *Fig. 19a* illustrates the localisation in the stigma of *Hordeum bulbosum*, observed in this case after resin embedding and sectioning at 2–3 μm. Comparison with *fig. 19b* shows that the reaction product is truly extracellular.

4. DISCUSSION

The interpretation of the organisation of an organ as delicate as the grass stigma must perforce rely very heavily upon fine-structural and cytochemical data. This is notably true of the wall and its superimposed layers, which form a strikingly complex system. The outer pellicle ('A' in *fig. 1*) is presumed to correspond to the surface secretion present on other 'dry' stigmas (MATTSSON et al. 1974), which in all species tested has non-specific esterase activity (reviews, HESLOP-HARRISON, HESLOP-HARRISON & BARBER 1975; CLARKE & KNOX 1978). In his remarkable studies of the stigmas of marine angiosperms, PETTITT (1976, 1980) has shown that a corresponding esterase-rich layer is even present on the surface of the papillae of the stigmas of such species as *Thalassia hemprichii* (Hydrocharitaceae), which are immersed in sea water throughout their functional life. The surface of dry stigmas also binds various lectins (Y. HESLOP-HARRISON1976; KNOX et al. 1976; PETTITT 1980). In the grass *Phalaris minor*, the binding of Concanavalin A to the stigma papillae has been investigated electron microscopically (Y. HESLOP-HARRISON 1976); here it is quite clear that the lectin associates with the pellicle, indicating that the binding sites are in this layer (see also CLARKE & KNOX 1978).

In the earlier studies of the pellicle of the stigma papillae of *Phalaris*, we noted the presence of a layer some 90 nm in thickness intervening between it and the cuticle. The corresponding layer in rye ('B' in *fig. 1*) is of a similar thickness over the distal parts of the papillae, but, as we have seen, in clefts, and especially at the junction points with other cells, the layer is a great deal thicker and can be resolved with the optical microscope. The material composing the layer is a

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Figs. 10–14. Optical micrographs of 1–1.5 μm sections of resin-embedded, glutaraldehyde-fixed stigmas of *Secale cereale*.

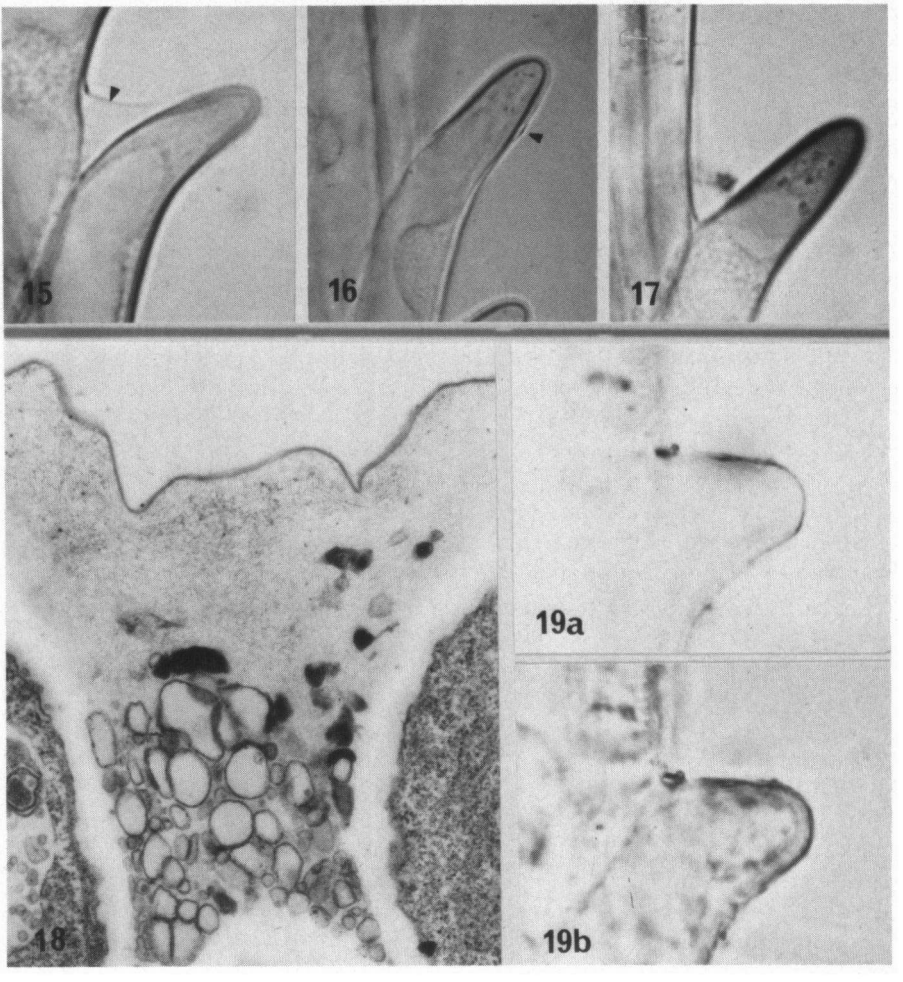
Fig. 10. Transverse section of secondary branch, fluorescence micrograph, calcofluor white staining. The staining is restricted to the wall layer E of *fig. 7*. × c. 1,500.

Fig. 11, as *fig. 10*, toluidine blue staining, micrograph taken with medium green filter. The interstitial material of wall layer D is stained metachromatically (pink in the original preparation); layer E orthochromatically. × c. 1,500.

Fig. 12. Centre zone of secondary branch, Nobel staining for protein. The interstitial material of the pollen tube transmitting tract (corresponding to wall layer D) is stained but not wall layer E. × c. 3,500.

Fig. 13. Longitudinal section of secondary stigma branch, fluorescence micrograph, calcofluor white staining. The section grazes the internal walls of the papillar cells, and the discontinuities in the wall layer E corresponding to the pitfields may be seen. × c. 1,500.

Fig. 14. As *fig. 13*, section grazing the outer papillar walls, which lack pitfields. × c. 1,500.



Figs. 15–17. Optical micrographs of intact, living stigma papillae of *Hordeum bulbosum*. These micrographs all show the reflexed tip region with the cytoplasmic content seen in section in the electron micrograph of *fig. 3*. All × c. 750.

Fig. 15. Surface secretion accumulation in the angle of a papilla and a secondary branch (arrow).

Fig. 16. Local secretion accumulation on the reflexed papilla tip (arrow).

Fig. 17. Neutral red 'vital' staining (2 min), showing the penetration into the tip zone of the papilla. Cyclosis was still evident in this papilla at the time of the micrograph.

Fig. 18. Electron micrograph of the junction region between a papilla and a secondary stigma branch, showing a large vesicle swarm in wall layer D. Uranyl acetate-lead citrate staining. × c. 26,000.

Fig. 19. Optical micrographs of a 2–3 μm section of a resin embedded stigma of *Hordeum bulbosum*, esterase localisation. × c. 1,400.

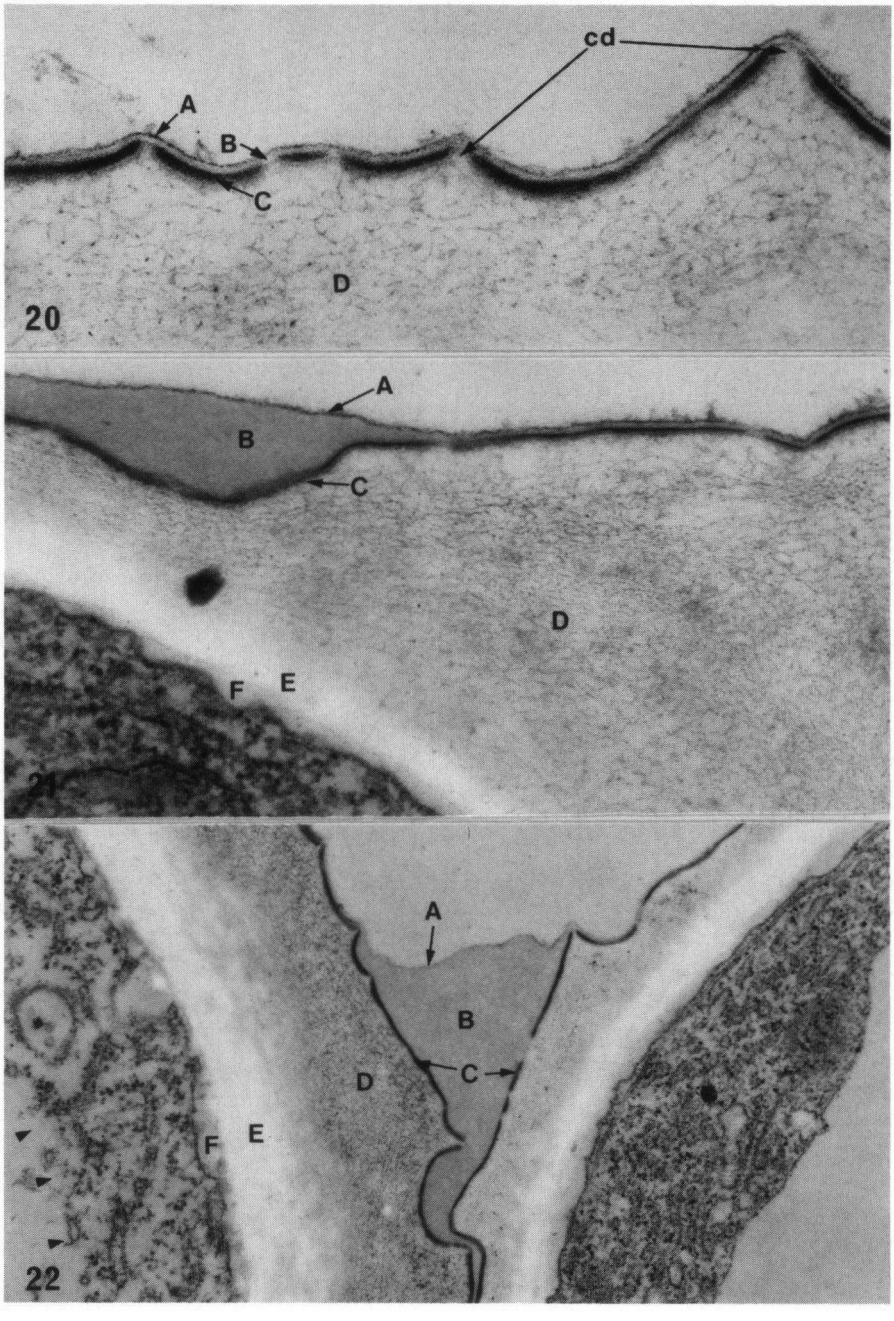
Fig. 19a, bright field. The reaction product is seen as an opaque layer on the papilla surface. Fig. 19b, phase contrast, showing the wall and cell contents.

viscous fluid, not dispersing readily in water, with staining properties suggesting a mucopolysaccharide content, but with no indication of a protein component. Evidently this layer is another secretion product of the stigma papilla.

The cuticle of the receptive parts of the papillae is invariably thin and discontinuous in dry-stigma species (HESLOP-HARRISON, HESLOP-HARRISON & BARBER 1975; HESLOP-HARRISON, KNOX, HESLOP-HARRISON & MATTSSON 1975), and the grasses conform to the general rule. The closest parallels to cuticles of this kind are found in various types of glandular trichomes, notably those of the enzyme secretory cells of the gland heads of certain carnivorous plant species (FAHN 1979). Presumably the surface secretions of the grass stigma papillae leave the cells through the cuticular discontinuities as the cells mature and the receptive state is reached. The permeability of the cuticle of the receptive zone is, of course, essential for the hydration of the pollen (J. HESLOP-HARRISON 1979), and it accounts for the ease with which dyes penetrate into the cell in this region.

The wall stratum immediately within the cuticle (the layer 'D' of *fig. 1*) and the intercellular material with which it has continuity in the axis of the stigma branch have a particular significance, since they provide the medium through which the pollen tubes pass after the cuticle has been penetrated (J. HESLOP-HARRISON 1978). There are no defined transmitting tissues in the secondary branches; indeed, each branch constitutes in itself a cuticle-bounded transmitting tract. The interstitial material and the sub-cuticular material of the outer wall layer D are therefore to be compared with the intercellular material of the transmitting tracts of species with massive styles of the solid type, such, for example as various species of the Solanaceae (KROH 1973; KROH & HELSPER 1974; SASSEN 1974; CRESTI et al. 1976). The cytochemical reactivities of the interstitial material in rye and *H. bulbosum* are quite compatible with the biochemical properties of the intercellular material described by KROH (1973) for *Petunia* transmitting tissue, indicating the presence of acidic pectic polysaccharides as a major constituent. The interstitial material of the mature stigma branches of the grasses also evidently contains a protein component, as judged from its staining properties. This is in accord with the findings of CRESTI et al. (1976) for the intercellular material of the transmitting tract of the style of *Lycopersicum peruvianum*, where a protein component is incorporated during the final stage of maturation.

The source of the protein fraction in the interstitial material of the secondary branches of the grass stigma must, presumably, be the contiguous cells. These have peripheral ribosomal endoplasmic reticulum during the final stages of maturation, although lacking dictyosomes in the active configuration. Vesicles of the kind described by CRESTI et al. (1976) in the cells of the transmitting tract of *Lycopersicum peruvianum* during maturation have not been observed in the grasses. On the other hand, numerous paramural bodies are present. CRESTI et al. (1976) mention the presence of 'lomasomes' during the earlier development of the *Lycopersicum* transmitting tract, suggesting a further homology. It remains a possibility that the paramural bodies constitute a form of granulocrine secretory system in the grass papillar cells, and that the vesicles observed in the interstitial material and the layer D of the outer wall are the means of delivering proteins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Figs. 20–22. Electron micrographs of the walls of stigma papillae of *Secale cereale*, standard uranyl acetate-lead citrate staining.

Fig. 20. Stratification of the outer zone; labelling as in *fig. 7*. The pellicle layer, A, is separated by layer B from the cuticle, C; in this stretch of the wall the layer B has a constant thickness of c. 110 nm. Cuticular discontinuities (cd) are apparent; the layers A and B continue over these uninterruptedly. The micrograph also shows the loose microfibrillar content of layer D. × c. 108,000.

Fig. 21. As *fig. 20*, section just below the junction point of the reflexed papilla tip with the secondary stigma branch. Where the secretion composing layer B accumulates in clefts, the pellicle layer is lifted away from the cuticle. × c. 70,000.

Fig. 22. As *fig. 21*, transverse section at the junction point; axis of the secondary stigma branch to the right. A continuous plasmalemma is absent from the reflexed part of the papilla to the left (arrow heads). × c. 33,000.

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into the pollen tube pathway.

The inner layer of the papilla wall, 'E' of *fig. 1*, is unusual in its remarkable electron transparency following standard post-staining procedures. No structure could be distinguished in it with the staining procedures used, and it is significant, also, that it shows no birefringence. Thus, while the cytochemical properties, including the Thiery reactivity, clearly indicate a polysaccharide composition and the strong affinity for calcofluor white may reveal the presence of 1,4-linked glucans (MAEDA & ISHIDA 1976), there is no satisfactory evidence of a microfibrillar cellulosic component. The intense staining with PTA at low pH might be taken to indicate the presence of hemicelluloses and pectic components, but in view of the uncertainty as to what this staining procedure actually reveals in plant cells, it seems premature to draw any conclusions.

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