CHAPTER SIX

INFECTION OF WHEAT AND *BROMUS DIANDRUS* BY *PYRENOPHORA SEMENIPERDA:* THE INFECTION PROCESS

6.1 Introduction

None of the research on *P. semeniperda* undertaken to date has described the various stages of the infection process. When considering an organism as a potential bioherbicide, it is necessary to understand the infection process and the host-pathogen interaction. This necessitates determining which plant tissues become infected, the timing of the phases of infection and the identification of any mechanisms of resistance found in the crop or the target weed. Knowledge of the host tissues which may be infected by the pathogen is essential, since infected tissue which remains asymptomatic may still have a negative effect on plant growth. This is important when one is screening different supposedly 'non-host' crop species. Understanding the timing of the phases of host infection is also necessary to optimise infection, and to determine the most conducive environmental conditions for infection. Mechanisms of host resistance must be studied because the efficacy of a bioherbicidal candidate may be enhanced by overcoming the constraints to infection through application technique or bioherbicide formulation.

The research described in this chapter was designed to:

a) give an account of the time sequence for each stage of the infection process;

b) examine the infection process on a wide variety of host tissues;

c) obtain evidence of any mechanisms of host resistance;

d) compare the infection process of *P. semeniperda* with that of literature reports of other related *Pyrenophora* spp..

6.2 Materials and Methods

6.2.1 The infection process on leaves.

The sequence of events leading to infection by *P. semeniperda* (isolate 580681) on seedling and adult leaf pieces of wheat and *B. diandrus* was studied using both light and electron microscopy.

Ten day old seedlings and adult plants of both wheat and *B. diandrus* were inoculated with a conidial suspension of 1 x 10^3 conidia ml^{-1} and immediately placed into a dark dew chamber at 20 ± 1 °C for an incubation period of 3, 6, 9, 12, 15, 18, 21, 24, 36 or 48 h. Surfactants were not used to aid wetting of leaves because they have been reported to induce papillae and halo production in cereal leaves (Hargreaves, 1982). After incubation, plants were removed from the dew chamber and placed in front of a fan for 10 min to remove dew from leaves. In the case of seedling plants, leaf pieces (approx. 1.5 cm in length) were excised from the centre of the first true leaf. Adult leaves were sampled by excising leaf pieces (approx. 1.5 cm in length) from the centre of the flag leaf, and each of the next two lower leaves. All leaf segments were prepared for examination with the light microscope by using the whole leaf clearing and staining technique of Keane, Limongiello & Warren (1988) or prepared for scanning electron microscopy (see Appendix 2).

The experiment consisted of five replicates per time period arranged in a randomised complete block design within the glasshouse (pre-inoculation) and in the dew chamber (post-inoculation). A replicate consisted of five seedling or three adult plants.

For studies involving light microscopy, each leaf piece was mounted in 50 % glycerol and examined under 100 to 1000 times magnification using brightfield light. For scanning electron microscopy, leaf pieces were examined at 100 to 10000 times magnification using a Jeol JSM-35 scanning electron microscope at the Electron Microscope Unit at the University of New England. The first 20 conidia encountered per leaf piece were recorded as either having germinated or not. Germination was considered to have occurred if the germ tube was longer than the width of the conidium from which it had arisen. The first 20 conidia per leaf piece which had germinated were examined for the production of appressoria and infection hyphae, and the presence or absence of these structures was noted. Due to the nature of scanning electron microscopy only pre-penetration structures of *P. semeniperda* were observed using this technique.

Photomicrographs of the infection process were taken with a Zeiss MC-35 camera mounted on a Kyowa Unilux-12 microscope loaded with Fujicolor 100 Tungsten biased film. Electron micrographs were taken with a Mamiya camera loaded with Kodak TMX 6502 film.

6.2.2 The infection process on floral tissue.

The infection process of *P. semeniperda* was examined on floral tissue of wheat using light and electron microscopy.

Wheat plants were inoculated at anthesis (GS 60) by dipping the inflorescences into a conidial suspension (1 x 10^3 conidia ml^{-1}) and placing the plants in a dark dew chamber at 20 ± 1°C for 24 h. After incubation, the plants were removed from the dew chamber and placed in front of a fan for 10 min to remove dew. Individual florets were then dissected from the inflorescences, and further dissected into groups of paleas, lemmas, ovaries, and stamens and anthers. Each group was then prepared for light microscopy using the whole leaf clearing and staining technique or electron microscopy as described previously.

In a second study, the plants were placed into the glasshouse after incubation for a further 14 days. Developing caryopses were fixed in 70 % ethanol for 48 h, dehydrated through a tertiary butyl alcohol series, and embedded in paraffin. Embedded specimens were softened for 48h in a 10 % glycerol solution with 1 % sodium lauryl sulfate (Lawrence, Nelson & Ayers, 1981). Serial sections (10µm thick) were cut with a rotary microtome, deparaffinized, and stained with safranin and light green.

In a third study, groups of floral tissues were dissected prior to inoculation. These groups were placed on filter paper moistened with sterile distilled H_2O contained in Petri plates. The floral parts were inoculated by applying a conidial suspension with a handheld atomiser until incipient run-off. The Petri plates were then sealed and incubated in a dark incubator set at 20 ± 1°C for 24 h. Each group was then prepared for electron microscopy.

In each of the three studies. 10 individual florets were dissected from each of five replicate plants. Therefore, the floral tissue from a total of 50 florets was examined in each study.

The floral material was prepared for examination and examined in the same way as the leaves.

6.3 Results

6.3.1 The infection process on leaves.

At 3 h after inoculation, conidia had germinated to produce two germ tubes with an average length of 16 μm. The majority of conidia germinated from both polar cells. Germination from the intermediate cells was rare. Germination from both polar and intermediate cells of the same conidium was never observed. The formation of appressoria was observed at 3 h after inoculation. However, host penetrations were not visible at this time (Fig 6.1, Table 6.1). The proportion of conidia that had germinated reached maxima at 12 h and 18 h after inoculation on seedling and adult leaves respectively. This was the case on both wheat and *B. diandrus*. The proportion of conidia that germinated was always lower on adult leaves. In a large majority of cases, germinated conidia produced germ tubes that branched. As a result, most conidia initiated multiple penetration sites (Fig.6.2). In some instances, pieces of hyphae and conidiophores were observed to germinate and produce infection structures (Fig 6.3).

The proportion of germinated conidia that produced appressoria was maximal at 21 h after inoculation for both species and leaf types. However, five times as many appressoria were produced on the surface of seedling leaves than on adult leaves. Appressoria were produced on the germ tubes either terminally, intercalarially or as hyphopodial-like structures (Fig. 6.4). Appressoria that formed terminally on germ tubes were sharply delimited from the germ tube by a septum (Fig. 6.4). On wheat leaves, appressoria were produced over epidermal cell wall junctions and occasionally, over epidermal cells, stomatal guard cells and trichomes (Fig. 6.4). On leaves of *B. diandrus,* appressoria were produced over stomata and rarely over epidermal cell wall junctions (Fig. 6.5). Appressoria were often associated with the production of extra-cellular sheath-like material (Fig. 6.5).

Sites of attempted penetration on both wheat and *B. diandrus* were distinguishable by the presence of halos, which were first observed at 12 h after inoculation. These halos were easily observed because they were stained differentially and sharply delimited from the uninfected host tissue.(Fig. 6.6). Papillae were produced in response to host cell invasion by *P. semeniperda* in both plant species. Papillae began as aggregates of dense cytoplasm and finally appeared as densely stained protrusions beneath appressoria (Fig. 6.7). Papillae were produced adjacent to appressoria on the inner epidermal cell wall surfaces. Infection hyphae

Table 6.1. The time sequence of events during the infection process of *P. semeniperda* on seedling leaves of wheat and *B. diandrus*.

	Wheat*								*B. diandrus* *							
	% Germ		% App		% Infect		% H C N		% Germ		% App		% Infect		% H C N	
Hours	S[a]	A[a]	S	A	S	A	S	A	S	A	S	A	S	A	S	A
3	10.5	3	1	0	0	0	0	0	9.8	3.3	0	0	0	0	0	0
6	20.6	8.2	3	0	0	0	0	0	21.1	9.1	2.7	0	0	0	0	0
9	50.1	12.9	5	1	0	0	0	0	46	11	4	0	0	0	0	0
12	99	20.7	9	2.4	3	0	0	0	98	22	7.6	1.5	2.4	0	0	0
15	99	21	18	3	4	0	0.9	0	99	22	20.2	3.7	3.1	0	0.5	0
18	99	28	21	4.0	4.3	0.6	1	0	99	24	20.5	3.9	3	0.2	0.7	0
21	99	28	22	4.2	5	0.9	1.2	0	99	24	21.3	4.3	4.6	0.3	1	0
24	99	28	22	4.2	5	1.1	1.8	0.1	99	24	21.3	4.3	4.6	0.9	1.5	0
36	99	28	22	4.2	5	1.1	1.8	0.1	99	24	21.3	4.3	4.6	0.9	1.5	0
48	99	28	22	4.2	5	1.1	1.8	0.1	99	24	21.3	4.3	4.6	0.9	1.5	0

*Germ- is the proportion of conidia that germinated, App- is the proportion of germinated conidia that produced appressoria, Infect- is the proportion of germinated conidia that produced infection hyphae, and HCN- is the proportion of germinated conidia that were associated with host-cell necrosis.

[a] S-is inoculated seedling leaves and A is inoculated adult leaves.

of *P. semeniperda* failed to penetrate through papillae and growth of the pathogen ceased.

Approximately 25 % of the sites of attempted penetration were not associated with papillae and the infection hyphae were able to ramify intercellularly through the host tissue. Successful penetration of seedling leaves of wheat and *B. diandrus* first occurred 12 h after inoculation. Penetration of adult leaves of both species was first observed at 18 h after inoculation. However, successful penetration of seedling and adult leaves of either grass species was only initiated by approximately 5 and 1 % of germinated conidia respectively. The first infection structure that developed was an intracellular vesicle that formed one or two infection hyphae (Fig. 6.8). Infection hyphae were not observed to penetrate host cells, but rather grew through the intercellular spaces. Host cells often collapsed prior to contact with infection hyphae.

Host-cell necrosis was clearly visible in whole-leaf mounts as collapsed and darkly stained tissue (Fig. 6.9). The proportion of germinated conidia that produced germ tubes that penetrated the host and later became associated with host-cell necrosis was about 1 - 2 % in the seedling leaves of both grass species. Host-cell necrosis in adult leaves of wheat was observed in 0.1 % of infections derived from germinated conidia and none was observed in adult leaves of *B. diandrus*.

6.3.2 The infection process on floral tissue.

Conidia germinated, produced germ-tubes and subsequent infection structures on all of the floral tissues tested.

The infection process on the lemmas and paleas of wheat was similar to that on seedling leaves. Germination of a conidium was followed by the production of several appressoria which gave rise to infection hyphae within the intercellular spaces. Cell wall appositions in the form of papillae were also present at some sites of attempted penetration.

Anthers of wheat florets were not observed to become infected by *P. semeniperda*. Infection was limited to the pre-penetration processes. Following germination of conidia, germ tubes grew randomly across the epidermal surface of the anther (Fig. 6.10). Occasionally, a hyphopodium was produced, but penetration of the host tissue was never observed. At 48 h after inoculation, hyphae had grown over a large proportion of the anthers' surface.

On ovarial tissue, the germ tubes and subsequent surface hyphae grew saprophytically on the stigmatic and stylar tissue, but never produced appressoria or other infection structures (Fig. 6.11). However, appressoria and hyphopodial-like structures were formed on the cell wall junctions of the ovary (Fig. 6.12). Penetration of the ovary wall also occurred without the formation of appressoria. In these cases, germ tubes penetrated the ovary wall without any noticeable swelling, particularly where wounding of the epidermis was apparent (Fig. 6.12).

Infection hyphae were generally confined to the epidermal and middle layer of the developing caryopsis where they ramified through the intercellular spaces. The integumentary layer was a barrier to growth and hyphae rarely penetrated this band of cells (Fig. 6.13). Hyphae were not observed in the nucellus, micropyle or the embryo sac of the developing seed.

Stroma of *P. semeniperda* was observed to erupt from the seed coat of wheat caryopses and was confined by the aleurone layer of the seed (Fig. 6.14).

6.4 Discussion

Germination of conidia of *P. semeniperda* was predominantly from polar cells, as has been reported for other species of *Pyrenophora* eg. *P. teres* (Van Caeseele & Grumbles, 1979; Coyle & Cooke, 1993) *P. graminea* (Smedegård-Peterson, 1976) *P. avenae* (Arora, Mandahar & Pahwa, 1980) and *Pyrenophora dictyoides* Paul & Parberry (Cromey & Cole, 1985). However, Larez, Hosford Jr. & Freeman (1986) reported that germ tubes of *P. tritici-repentis* were produced from both polar and intermediate cells of the same conidium.

Germination of conidia of *P. semeniperda* reached a maximum 12 h after inoculation and then plateaued on seedling and adult leaves of both *B. diandrus* and wheat. This is slightly longer than that reported for other species of *Pyrenophora.* Larez *et al.* (1986) reported that 65 % of conidia had germinated by 3 h and 95 % by 6 h after inoculation. Van den Berg & Rossnagel (1990) reported a similar finding for *P. teres*, whilst germination of *P. avenae* was reported to be maximal after 4 h (Arora *et al.*, 1980).

Why a much smaller proportion of *P. semeniperda* conidia germinated on adult leaves of both wheat and *B. diandrus* is uncertain. It may have been due to physiological or morphological differences between seedling and adult leaves. The waxy nature of the cuticle of adult leaves rendered the leaves hydrophobic. Although the presence of free water has not

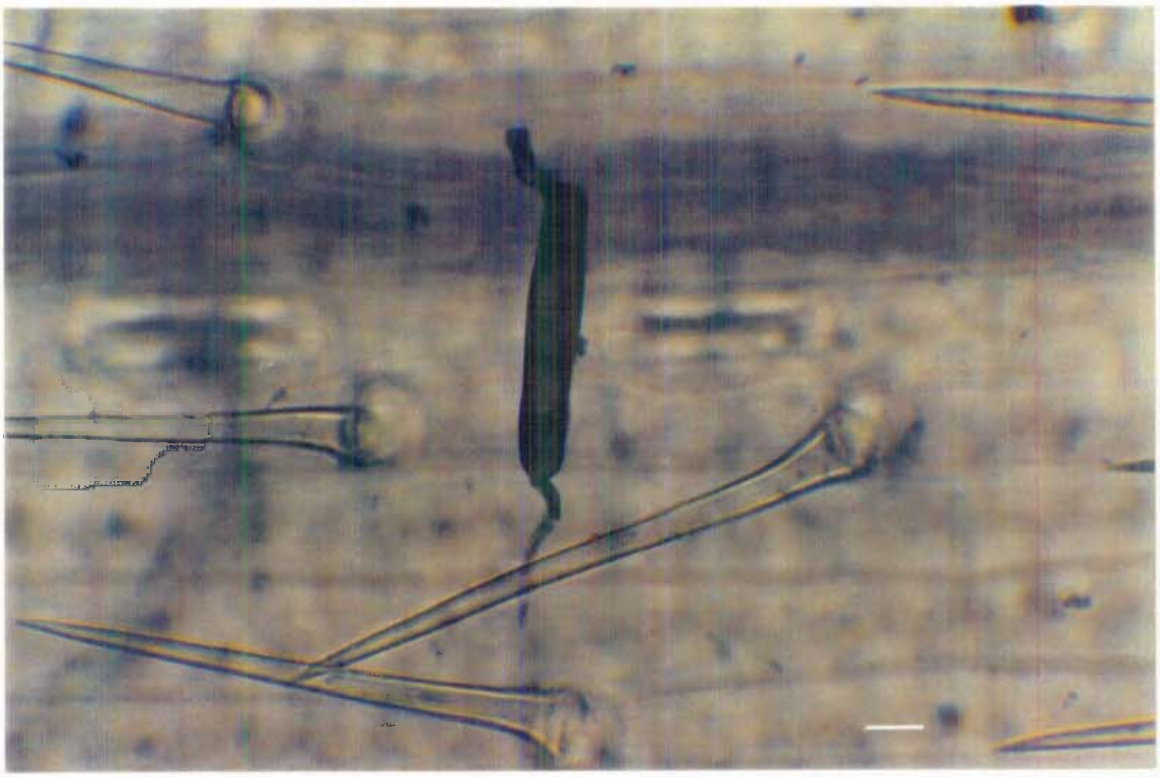

Fig. 6.1. Photomicrograph of germinating conidium of *P. semeniperda* on a seedling wheat leaf 3 h after inoculation. The bar in the photograph represents 10 μm.

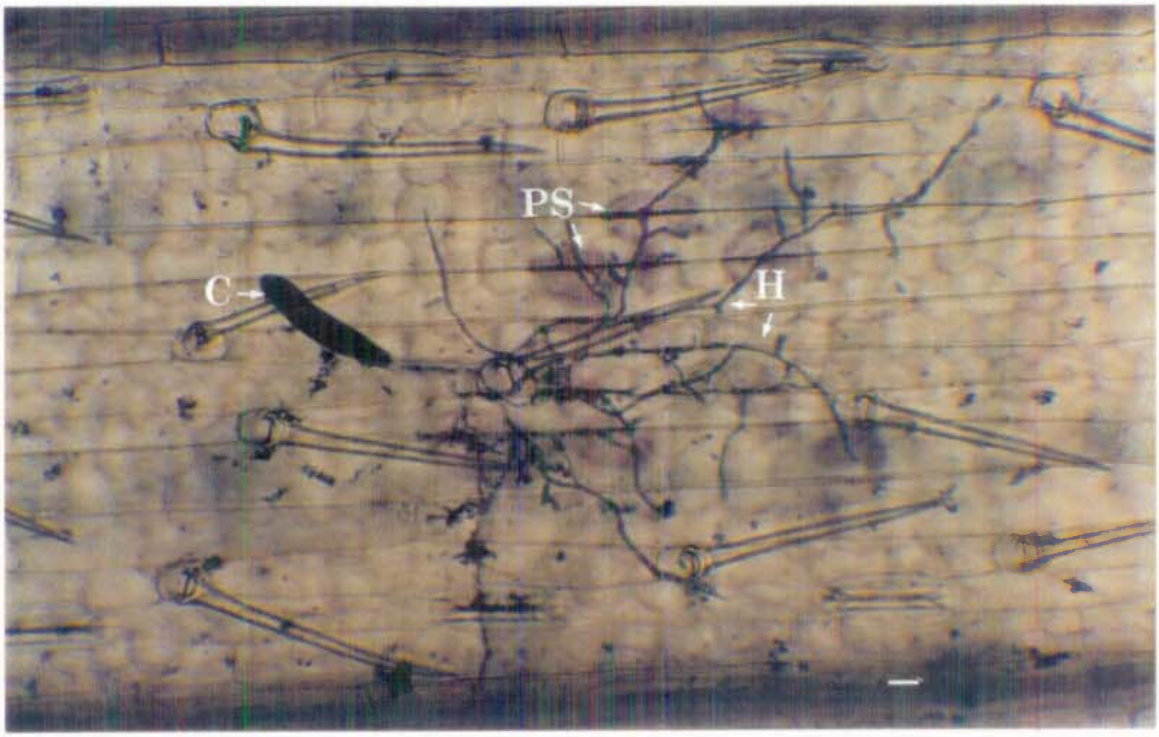

Fig. 6.2. Photomicrograph of *P. semeniperda* germinating conidium (C) with branched surface hyphae (H) and multiple penetration sites seen as haloes (PS). The bar in the photograph represents 10 μm.

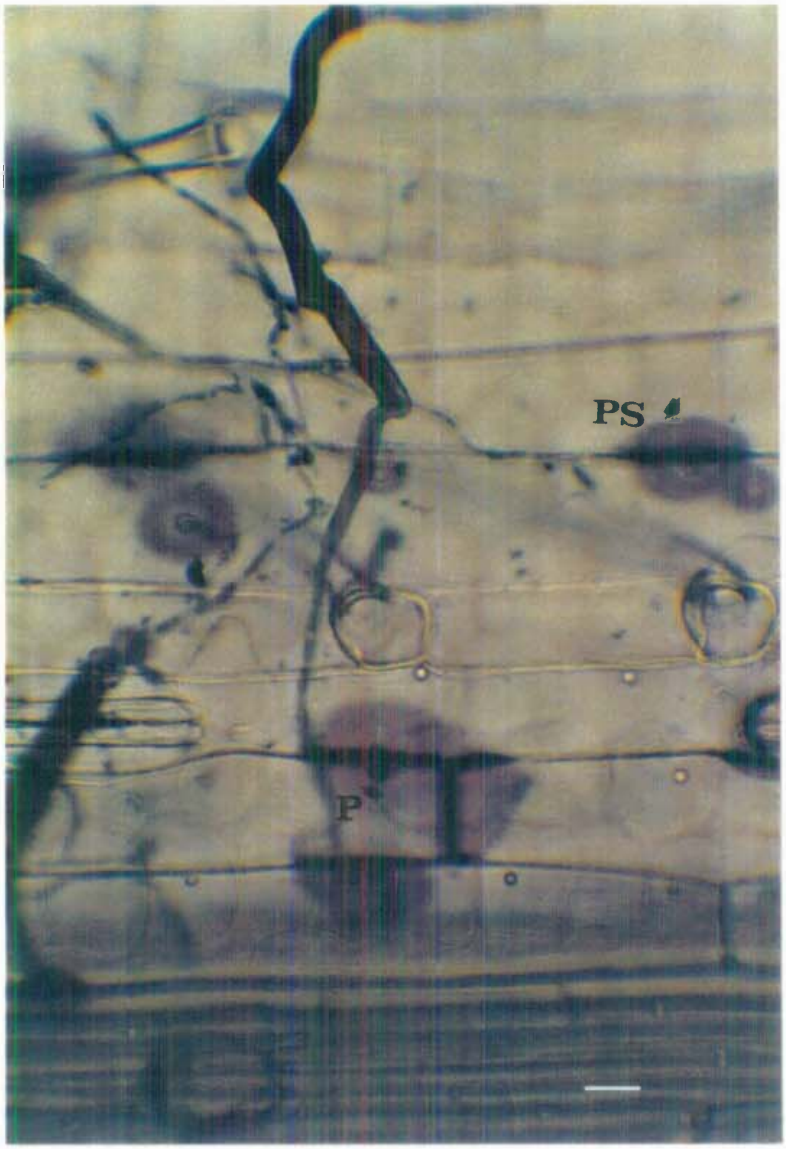

Fig. 6.3. Photomicrograph of germinated conidiophore segment of *P. semeniperda.* Note the penetrations sites (PS) and papilla (P). The bar in the photograph represents 10 µm.

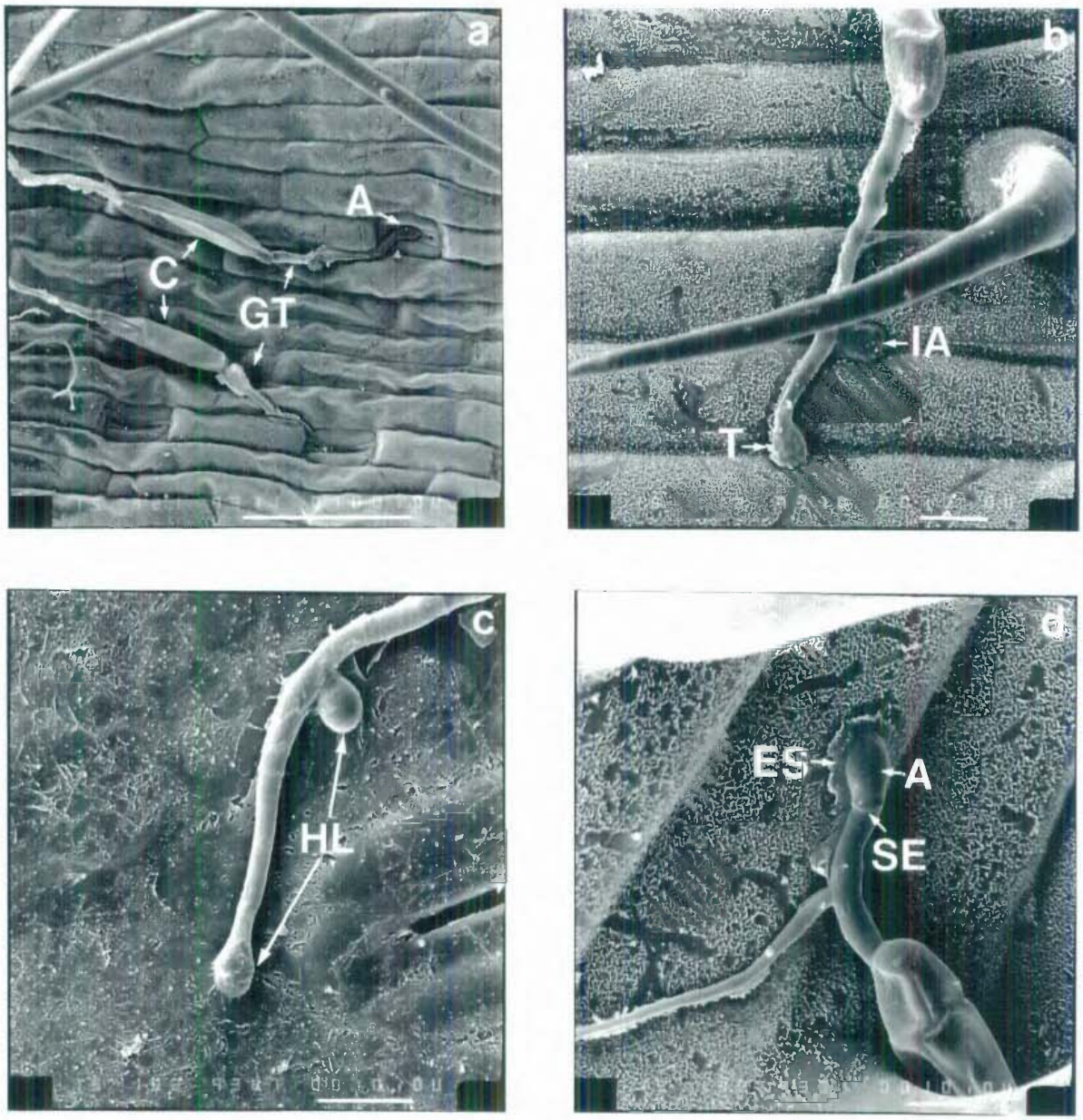

Fig. 6.4. Electron micrographs of *P. semeniperda* showing various appressoria morphologies and modes of penetration.

a) Possible stomatal penetration of a leaf of *B. diandrus* by *P. semeniperda* at 24 h after inoculation (Bar = 100μm). Note conidia (C), germ-tubes (GT) and appressoria (A).

b) Terminal (T) and intercalary appressoria (IA) formed over anticlinal epidermal cell walls and possible penetration of a wheat leaf by *P. semeniperda* at 18 h after inoculation (Bar = 10 μm).

c) Hyphopodial-like structures (HL) formed by *P. semeniperda* on glume tissue of wheat at 24 h after inoculation (Bar = 10 μm).

d) Possible penetration site by *P. semeniperda* directly over epidermal cell *on B. diandrus* seedling leaf at 24 h after inoculation. Note appressorium (A) delimited by a septum (SE) and extra-cellular sheath (ES) (Bar = 10μm).

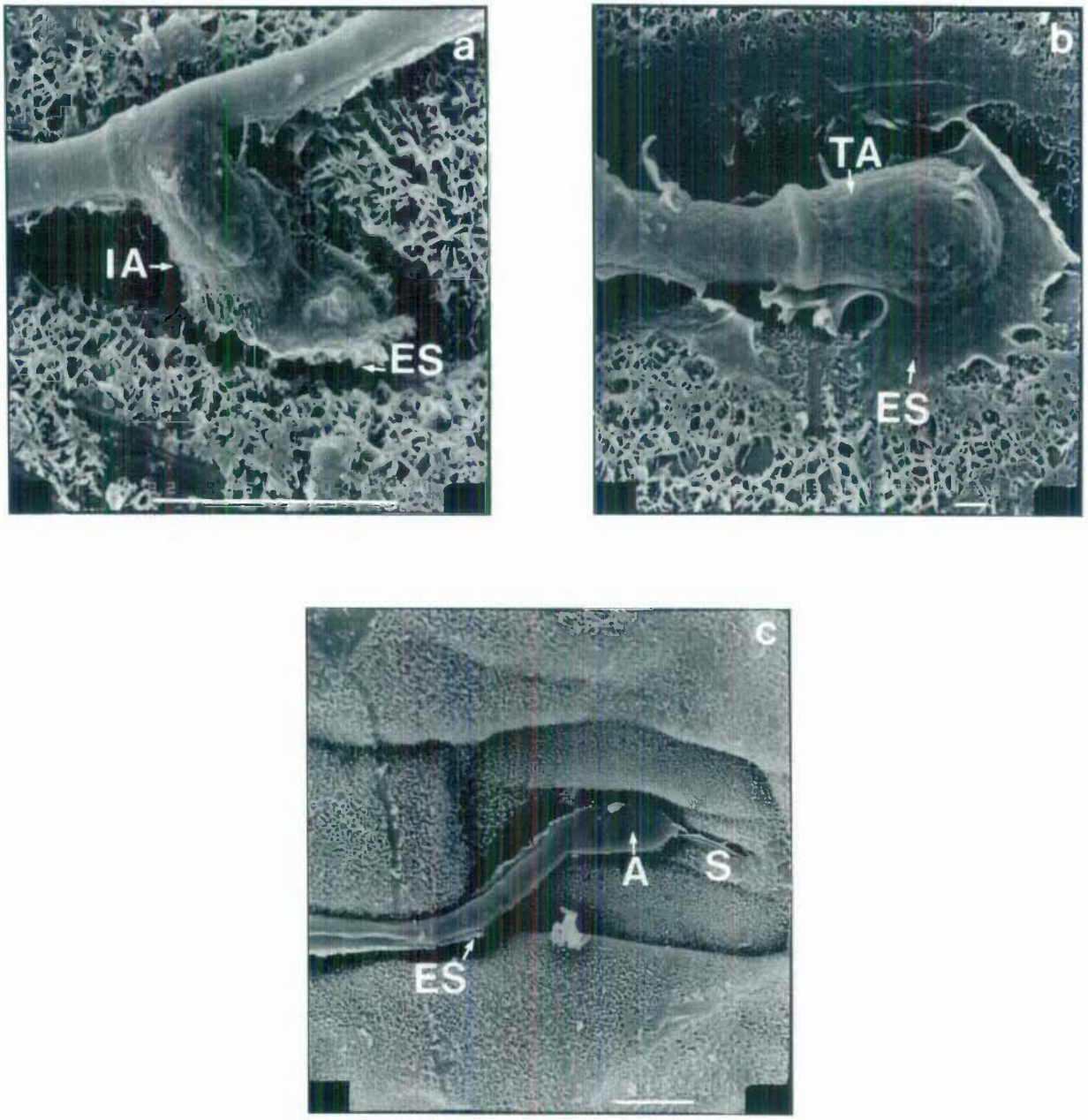

Fig. 6.5. Appressoria of *P. semeniperda* showing extra-cellular sheath material on seedling leaves of wheat and *B. diandrus*.

(a) Intercalary appressorium (IA) with extra-cellular sheath (ES) formed on wheat at 24 h after inoculation. The bar in the micrograph represent 10 μm.

(b) Terminal appressorium (TA) with extra-cellular sheath (ES) formed on wheat at 24 h after inoculation. The bar in the micrograph represent 1 μm.

(c) Appressorium (A) with extra-cellular sheath (ES) formed over stomate (S) on *B. diandrus* at 24 h after inoculation. The bar in the micrograph represent 10 μm.