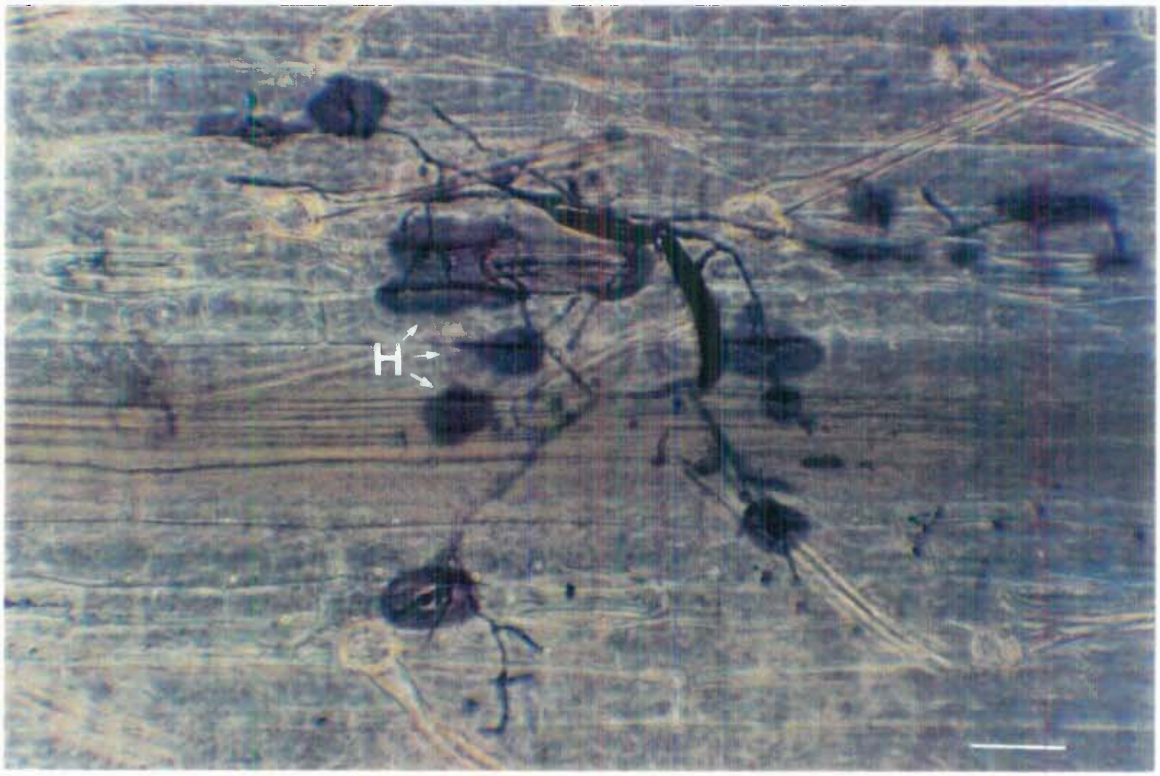


**Fig. 6.6.** Photomicrograph of multiple penetration sites of *P. semeniperda* with haloes (H) on a seedling leaf of wheat at 36 h after inoculation. The bar in the photograph represents 20 μm.

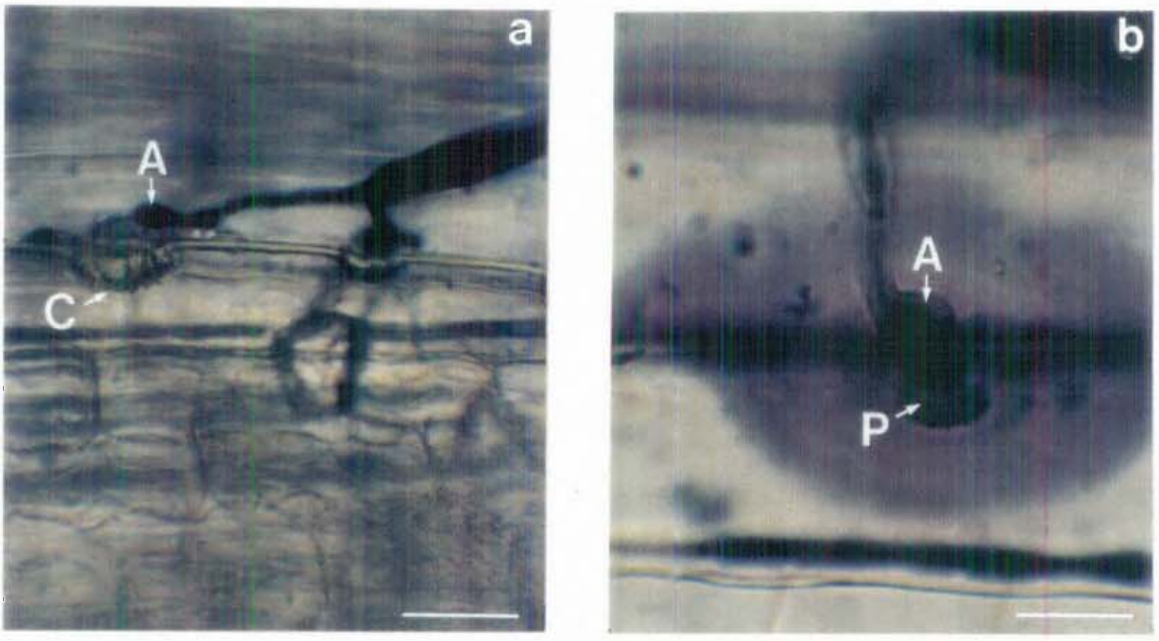


**Fig. 6.7.** Papillae formation in seedling leaves of wheat in response to penetration by *P. semeniperda* at 24 h after inoculation. The bars in the photographs represent 10 μm.

a) Note the dense cytoplasmic aggregates (C) beneath appressoria (A).

b) Note the densely stained young papilla (P) beneath the appressorium (A).

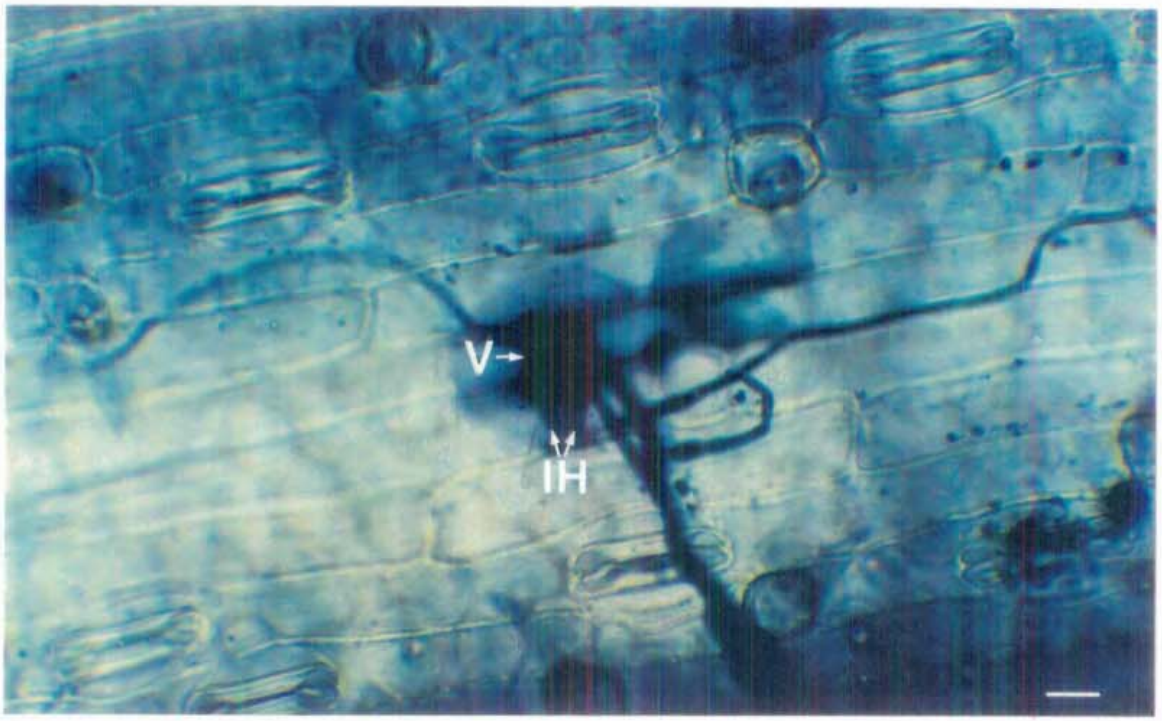


**Fig. 6.8.** Photomicrograph of penetration of a seedling leaf of wheat by *P. semeniperda* at 18 h after inoculation. Note the infection vesicle (V) and two infection hyphae (IH). The bar in the photograph represents 10 µm.

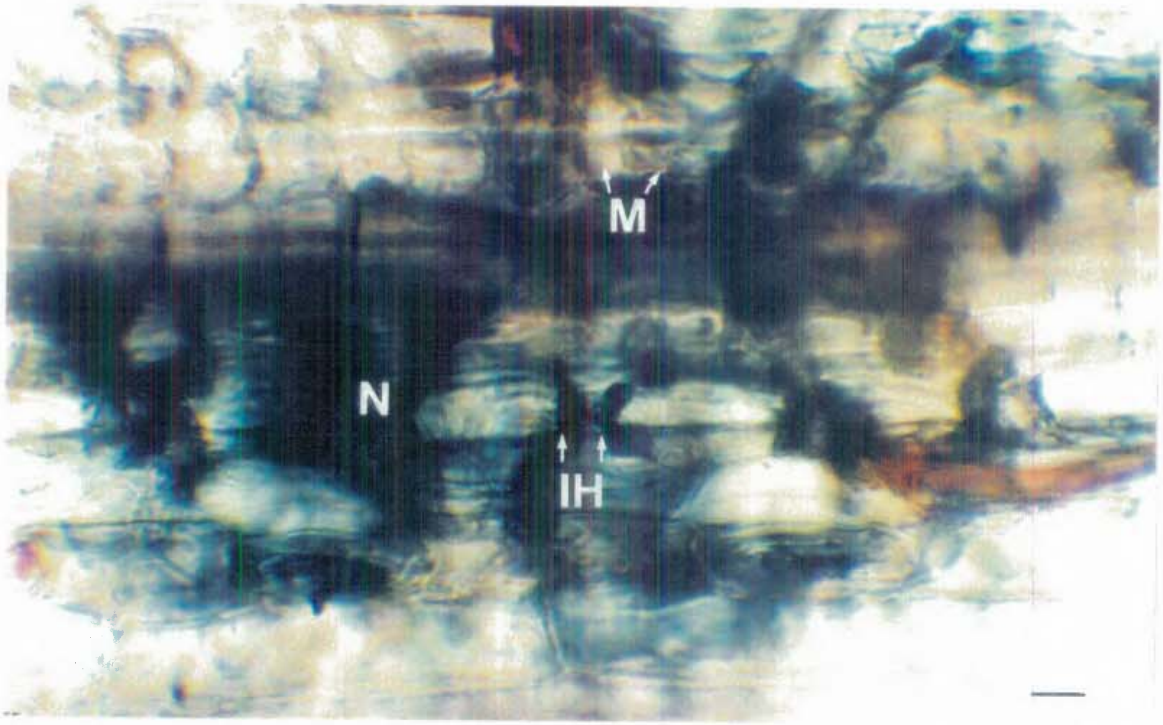


**Fig. 6.9.** Photomicrograph of host-cell necrosis in seedling leaves of wheat caused by infection with *P. semeniperda* at 48 h after inoculation. Note the infection hyphae (IH), collapsing mesophyll cells (M) and densely stained necrotic tissue (N). The bar in the photograph represent 10 µm.

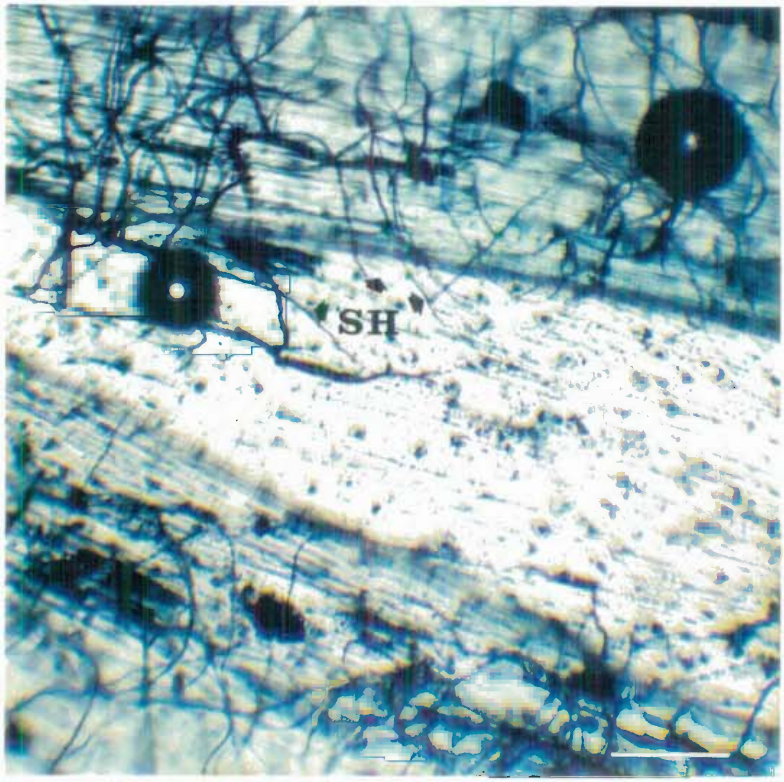


**Fig. 6.10.** Photomicrograph of saprophytic growth of surface hyphae (SH) of *P. semeniperda* on an anther of wheat at 48 h after inoculation. The bar in the photograph represents 50 μm.


**Fig. 6.11.** Electron micrograph showing saprophytic growth of surface hyphae (SH) of *P. semeniperda* on stigmatic tissue of wheat at 24 h after inoculation. The bar in the photograph represents 10 μm.

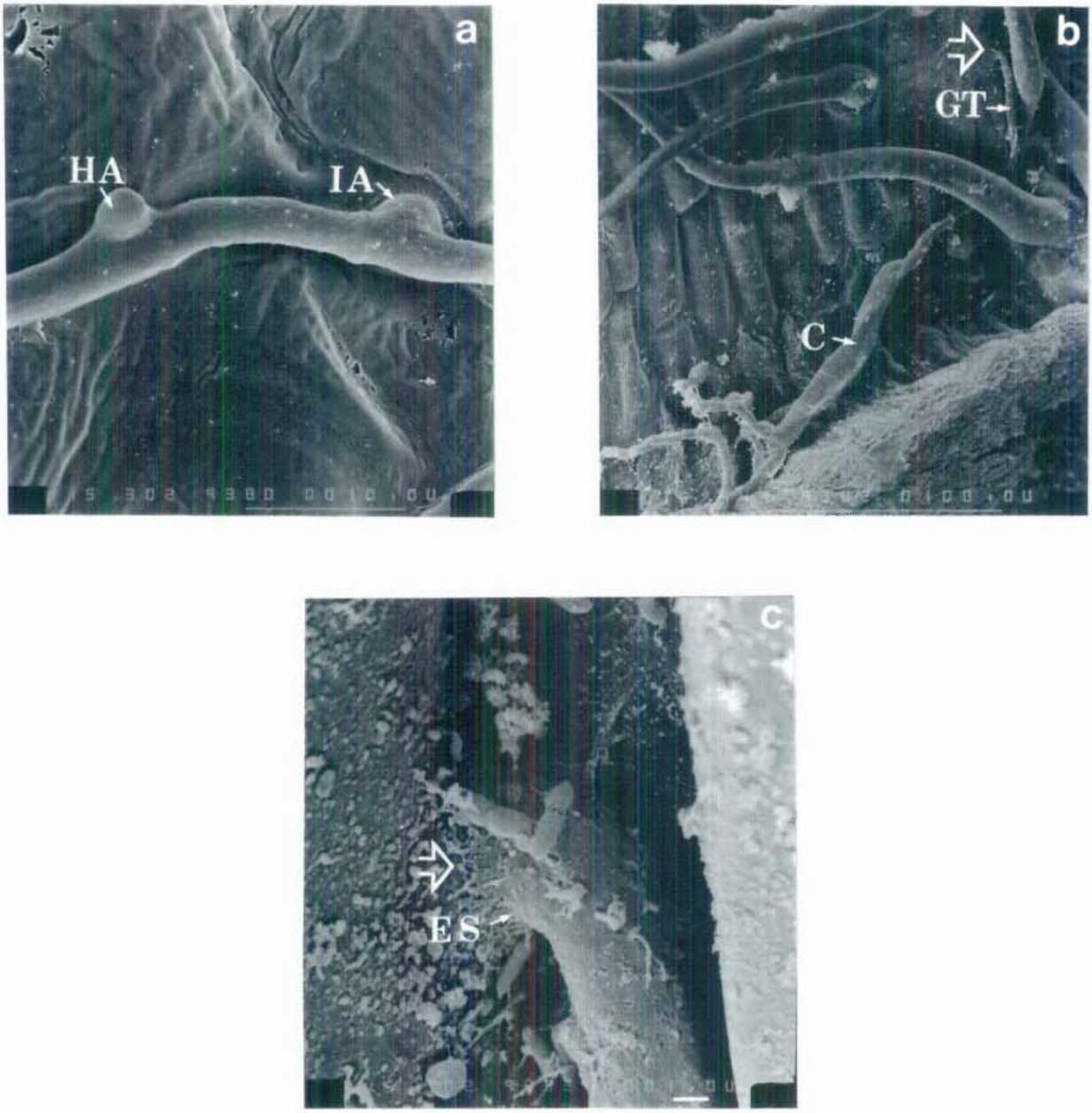


**Fig. 6.12.** Electron micrographs of infection of wheat ovary tissue by *P. semeniperda* at 24 h after inoculation.

a) Intercalary appressorium (IA) and hyphopodial-like structure (HA) formed on the surface of an ovary. The bar represents 10 μm.

b) Possible direct penetration of an ovary by *P. semeniperda*. Note the conidium (C) and germ-tube (GT). The bar represents 100 μm.

c) Close-up view of the possible penetration site in Fig. 6.12 (b). Note the extra-cellular sheath (ES). The bar represents 1 μm.

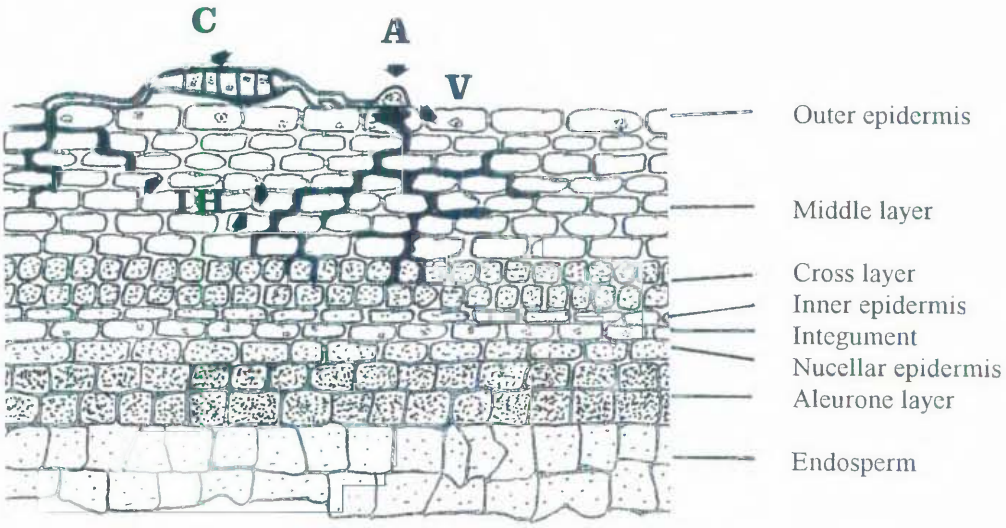


**Fig. 6.13.** Schematic representation of the infection of developing wheat caryopsis by *P. semeniperda* at 14 days after inoculation (approx. 400 times magnification). Note the conidium (C), appressorium (A), intracellular vesicle (V) and infection hyphae (IH).

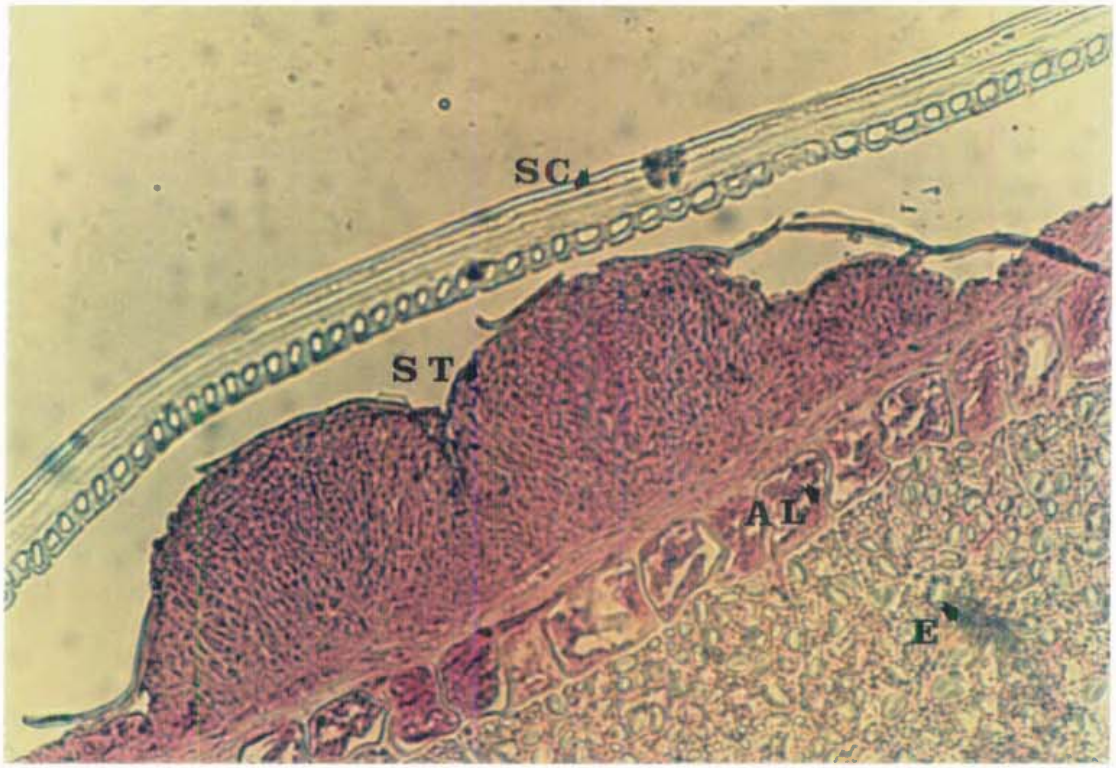


**Fig. 6.14.** Erumpent stroma (ST) of *P. semeniperda* in mature wheat seed. Note the seed coat (SC), aleurone layer (AL) and endosperm (E). Approximately 200 times magnification.

been determined as a pre-requisite for infection by *P. semeniperda* it seems a very likely proposition that it is the case. Thus, the hydrophobic nature of adult leaves may have deprived the spores from access to free water. Shaw (1986) reported that conidia of *P. teres* only germinated in the presence of liquid water. Similarly, all the other species of *Pyrenophora* infecting cereals require free water for conidial germination (Larez *et al.*, 1986). Therefore, it would appear that the decrease in overall germination of conidia of *P. semeniperda* on adult grass leaves may be due to a lack of free water rather than any other resistance mechanisms.

Germ tubes of *P. semeniperda* did not appear to show a thigmotropic response to the wax lattice of wheat leaves as was reported for *P. graminis tritici* by Lewis & Day (1972). Germ tubes appeared to grow randomly across the surface of all the tissue structures studied. Germ tubes were often observed to branch and produce many sites of infection from a single conidium. This has also been shown for all the major cereal infecting species of *Pyrenophora* (Van Caeseele & Grumbles, 1979; Arora *et al.*, 1980; Larez *et al.*, 1986; Coyle & Cooke, 1993). Germ tubes and surface hyphae of *P. semeniperda* grew saprophytically on anthers, stigmas and stylar tissue of wheat flowers. This is likely to have been due to the high content of sugars and proteins on the surfaces of these tissues (Alberts *et al.*, 1983).

Hyphal fragments of *P. semeniperda* were observed to germinate and produce infection structures. This is a particularly important finding because it gives evidence that hyphae can be used as infective propagules. The use of hyphal fragments as infective propagules would be particularly attractive because of the ease of production of large amounts of mycelial inoculum under liquid culture (see Chapter 3).

Appressoria of *P. semeniperda* were produced by germ tubes and surface hyphae either terminally, intercalarially or as hyphopodial-like structures. The morphology of appressoria of *P. semeniperda* ranged from slightly swollen germ tube apices to club-shaped structures. The shape of the appressoria appeared to be independent of the host tissue on which they were formed. Appressoria of *P. tritici-repentis* and *P. teres* have been described as club-shaped or round (Larez *et al.* 1986; Coyle & Cooke, 1993). Intercalary appressoria have not been reported for other species of cereal infecting *Pyrenophora*. The formation of an extra-cellular sheath around appressoria of *P. semeniperda* was observed by scanning electron microscopy. However, it did not appear to be an essential component of the infection process on either wheat or *B. diandrus*. Extra-cellular sheaths are believed to function by assisting

adhesion of pre-penetration infection structures and by releasing cutinase and other enzymes which break down the cuticle and ce l wall of the host (Mendgen & Deising, 1993; Schäfer, 1994). The appressorium wall of *P. dictyoides* on *L. perenne* was replaced by extra-cellular sheath material to form a penetration pore which appeared to dissolve the host wall material prior to infection (Cromey & Cole, 1985). A similar process has been described for species of *Colletotrichum* (Mendgen & Deising, 1993). Extra cellular sheath material has been described for both *P. tritici-repentis* (Larez *et al.*, 1986) and *P. teres* (Van Caeseele & Grumbles, 1979). Although the role of the extra-cellular sheath produced by *P. semeniperda* was not studied in detail, it is possible that it could facili ate adhesion of the appressorium to the cuticle. Schäfer (1994) suggested that toxins may assist fungal penetration into the host and that extra-cellular sheaths may be involved in their prod uction. As outlined in Chapter 5, toxins may also play a role in the pathogenesis of *P. semeniperda* and the possibility exists that this toxin/s may be produced prior to penetration of the host in association with extra-cellular sheaths.

The production of appressoria by *P semeniperda* was first observed at 3 h after inoculation and was maximal at 18 h after inoculation, which was similar to *P. tritici-repentis* (Larez *et al.*, 1986). However, Larez *et al.* (1986) found that the number of appressoria increased with duration of wet period. Appressoria of *P. semeniperda* developed from 22 % of germinated conidia on seedling leaves and 4 % on adult leaves. The reason for this difference is uncertain, but may be related to relative water retention abilities or differences in the nature of the leaf surface as discussed above. Arora *et al.* (1980) reported that appressoria of *P. avenae* developed on about 30 % of germ tubes. Although Larez *et al.* (1986) did not express the formation of appressoria by *P. tritici-repentis* as a percentage of germinated conidia, they reported that an average of 180 appessoria developed per 100 conidia. In the present study, an average of 420 appressoria developed per 100 germinated conidia on seedling leaves.

On leaf tissue of wheat, appressoria were produced primarily over anticlinal epidermal cell walls and in some cases directly over epidermal cells. In contrast, on leaf tissue of *B. diandrus* appressoria were produced primarily over stomata and rarely over anticlinal epidermal cell walls. The reason for this difference in penetration sites is uncertain. Similar differences have been reported in the literature. Van Caeseele and Grumbles (1979) concluded that *P. teres* produced appressoria and penetrated epidermal cells directly, while Keon & Hargreaves (1983) reported that appressoria usually developed in the region close to

the anticlinal epidermal cell walls. Coyle & Cooke (1993) described stomatal penetration by *P. teres*. Appressoria of *P. tritici-repentis, P. dictyoides, P. avenae* , *P. graminea* and *C. carbonum* have been reported to develop on epidermal cell junctures and rarely over stomata (Murray & Maxwell, 1975; Smedegård-Peterson, 1976; Arora *et al.*, 1980; Cromey & Cole, 1985; Larez *et al.*, 1986). Many studies on the differentiation of rust germ tubes into appressoria have been reported which have elucidated the nature of a pathogens response to various surface stimuli. Allen *et al.* (1991) reported that 27 different species of rust fungi developed appressoria in a thigmotropic response to different micro-fabricated topographies. Grambow and Riedel (1977) suggested that the differentiation of infection structures of *P. graminis* was due to the biochemica environment prevailing at the stomata. It is possible therefore, that slight differences in the surface topography or biochemistry of the host tissue may account for the differences in penetration sites observed in the present study.

Appressoria of *P. semeniperda* developed on ovary tissue of wheat on the cell wall junctions in a manner similar to that observed on leaf tissue. However, direct penetration of the ovary was also observed where wounding of the ovarial wall had occurred. Embryonic infection by *U. tritici* (loose smut) in the developing caryopsis of wheat occurs as a result of direct penetration of the pericarp after the formation of an appressorium (Pederson, 1956; Shinohara, 1972). Djerbi (1971) reported that *Gibberella zeae* (Schwein.) Petch the causal fungus of ear scab and blight of wheat and maize penetrated the pericarp without the formation of appressoria. He also found that the fungus penetrated the roots of wheat with the formation of appessoria. Infection of wheat by the black point fungus, *Alternaria alternata* (Fr.) Keissler occurred through direct penetration of the ovary wall after appessorial formation (Bhowmik, 1969). *Cercospora sojina* Hara penetrated seeds of soybean (*Glycine max* (L.) Merr.) without the formation of appressoria by growing through pores and cracks in the seed coat and directly through hilar tracheids (Singh & Sinclair, 1985).

Attempted penetration of the leaves of wheat and other members of the family Poaceae is often accompanied by the formation of papillae (Ride & Pearce, 1979) and an alteration of the upper epidermal walls adjacent to attempted penetration sites may be evidenced by haloes or disc-shaped areas (Ride & Pearce, 1979: Russo & Pappelis, 1981). The function and structure of papillae and haloes has been the subject of considerable debate in the literature. The nature of the components of papillae and haloes appears to be related to the host in which they are formed. For example, Vance & Sherwood (1977) reported that the general

mechanism of resistance to fungal infection in *Phalaris arundinacea* L. (reed canarygrass) involved the formation of lignified appositional wall growths. Ride & Pearce (1979) described the formation of papillae in wheat leaves which contained lignin and tested positive for the presence of callose and cellulose. Seedlings of wheat which contain the Sr5 gene for resistance to the stem rust fungus accumulated lignin at hypersensitive reaction sites (Tiburzy & Reisener, 1990). In contrast, Mayama & Shishiyama (1978) detected UV-absorbing substances but not lignin in barley leaves inoculated with *Erysiphe graminis* DC. Similarly, Hargreaves (1982) found that lignin could not be detected in papillae formed in oat leaves as a response to infection by *P. teres*. However, he reported the presence of an unknown phenolic compound. The modes of action of papillae have been variously described as: an impermeable barrier which isolated the fungus from adjacent cells and 'starved' the hyphae of necessary nutrients; deposits which act as a physical restriction to hyphal growth; or appositions with antifungal components which inhibit hyphal growth (Hargreaves, 1982). Haloes have been reported to be resistance structures produced by the host in response to infection with a pathogen or structures produced by degradation of the substance of the cell wall by the pathogen (Ride & Pearce, 1979; Russo & Pappelis, 1981). Ride & Pearce (1979) postulated that haloes were areas of localised lignification which may also contain silicon. Furthermore, they suggested that the role of haloes was to slow down the penetration process until the papilla was fully formed. Conversely, Russo & Pappelis (1981) showed that haloes were the result of degradation of the pectin in the middle lamella of cell walls due to enzymatic activity. They concluded that the pathogen (*Colletotrichum circinans* (Berk.).Vogl.) utilized the metabolised pectin. Russo & Pappelis (1981) also found that haloes appeared sunken when viewed with scanning electron microscopy.

Papillae were formed at 75 % of the sites of attempted penetration by *P. semeniperda* in leaves of both wheat and *B. diandrus*. The papillae were produced directly adjacent to appressoria on the inner epidermal cell wall surfaces. Infection hyphae were able to ramify through the host tissue at sites where no papillae formed. A similar finding was reported by Van Caeseele & Grumbles (1979) for infection of barley leaves by *P. teres* and Larez *et al.* (1986) and Loughman & Deverall (1986) for infection of wheat by *P. tritici-repentis*. Loughman & Deverall (1986) also reported the formation of haloes at some sites of attempted penetration by *P. tritici-repentis*. Cromey & Cole (1985) described the formation of intramural hyphae within epidermal cells of *L. perenne* with and without the presence of

papillae. They concluded that intramural hyphae were an infection 'error' rather than a response to an active host defence mechanism. In the present study, papillae appeared to behave as a physical barrier to growth of the hyphae of *P. semeniperda* because hyphae were never observed to grow beyond the papillae.

Haloes were present at all penetration sites regardless of whether or not papillae were formed in response to infection by *P. semeniperda*. This suggests that the presence of haloes is attributable to a mechanism related to the pathogen rather than a host resistance mechanism because successful penetrations occurred regardless of whether haloes were present or not. The possibility exists that a toxic metabolite/s produced by the fungus were responsible for the degradation of the cell wall and the resulting haloes. Evidence for the presence of haloes was not found using scanning electron microscopy.

At sites of successful penetration of the host by *P. semeniperda,* infection hyphae ramified through the intercellular spaces of the mesophyll without penetrating the cell walls, although it is possible that at later stages of infection *P. semeniperda* may grow intracellularly. Host cells appeared to collapse prior to contact with infection hyphae. *P. semeniperda* appeared to behave as a necrotrophic pathogen. This mode of action suggests that a toxic metabolite plays a part in the pathogenesis of *P. semeniperda*. *P. tritici-repentis* and *P. teres* are both reported to grow intra-cellularly in the epidermal cells of their hosts whilst remaining inter-cellular in the mesophyll layers (Van Caeseele & Grumbles, 1979; Larez *et al.*, 1986; Loughman & Deverall, 1986). Toxins have been established as factors of pathogenesis for both *P. tritici-repentis* and *P. teres* (Smedegård-Peterson, 1976; Lamari & Bernier, 1989b). Keon & Hargreaves (1983) showed that penetration of mesophyll cells ocurred in late stages of barley infection by *P. teres*.

The production of intra-cellular vesicles as the first post-penetration structure by *P. tritici-repentis, P. teres* and *P. graminea* (Smedegård-Peterson, 1976; Van Caeseele & Grumbles, 1979; Larez *et al.,* 1986 Loughman & Deverall, 1986) also was observed in infection by *P. semeniperda.*

This is the first description of the infection process of *P. semeniperda* on wheat florets. The infection hyphae of *P. semeniperda* that had penetrated the ovarial wall of wheat florets ramified through the intercellular spaces but were confined to the epidermal and middle layer of the ovary. In contrast to this, *G. zeae* invaded the pericarp, seminal integument, proteic layer and the embryo when wheat flowers were inoculated at anthesis (Djerbi, 1971). Djerbi

(1971) demonstrated that seeds inoculated after flowering were generally confined to the pericarp. Mycelium of the loose smut pathogen *U. tritici* has been observed to cross the integumentary layers into nucellar tissue and enter the developing embryo (Shinohara, 1972). Youcef-Benkada *et al.* (1994) investigated the location of *P. teres* in seeds of barley after artificial inoculation at the time of flowering. They concluded that all parts of the caryopsis were infected by both forms (f. *teres* and f. *maculata*) of the pathogen. Hyphae of *P. graminea* and *C. sativus* grew along the style and infected the parenchymatous tissue of the glumes and the epidermis of the developing caryopsis, but never grew into the embryo or endosperm (Platenkamp, 1976; Neergard, 1979). Porta-Puglia & Montorsi (1982) isolated *P. avenae* from bracts, caryopses tissue and embryos of oats. It is possible that *P. semeniperda* could establish itself as dormant mycelium within the glumes and epidermis of the developing caryopsis in a manner similar to that of *P. graminea* given that the palea and lemma tissue of florets was infected in the present study (Platenkamp, 1976). Hyphae of *Colletotrichum truncatum* (Schw.) Andrus & Moore and *Phomopsis* spp. were restricted to the seed coat of soybean seeds (Kunwar, Singh & Sinclair, 1985). Similarly, *C. sojina* was only located in the seed coat of soybean seeds and was not found in the cotyledons or embryo (Singh & Sinclair, 1985). *P. semeniperda* rarely was observed infecting deeper tissue. However, the embryo may be infected if the developing seed was inoculated some time after flowering, when the external layer of the seminal integument is thinnest and where the micropyle presents a favourable opening to the penetration of mycelia (Djerbi, 1971). Stroma of *P. semeniperda* was observed to erupt from the seed coat of wheat, but was confined by the aleurone layer, providing further evidence that it does not deeply infect wheat seeds.

## 6.5 Summary

The infection process of *P. semeniperda* on seedling and adult leaves of wheat and *B. diandrus* and floral tissue of wheat was investigated. 99% of conidia germinated on seedling leaves of both plant species while on adult leaves only 20-30 % germinated. It is suggested that this is a result of the waxy nature of leaf surfaces on adult leaves. Appressoria were formed as an essential component of the infection process on leaf material and were formed over the anticlinal epidermal cell walls of wheat and over stomata of *B. diandrus*. Ovarial infection was observed to occur either with or without the formation of an appressorium.

Infection of the ovaries also occurred through cracks and wounds without the formation of an appressorium. Resistance to infection was associated with the formation of papillae. Haloes in host tissue were usually formed in response to infection by *P. semeniperda*. However, it is likely that these occurred because of a pathogenic mechanism which is probably related to the formation of a toxic metabolite. The first post-penetration structures formed were intracellular vesicles from which infection hyphae developed and ramified through the intercellular spaces of the mesophyll. Cellular disruption in advance of infection hyphae was observed indicating that *P. semeniperda* is a necrotrophic pathogen. This suggests that a toxic metabolite was produced by the pathogen. Infection hyphae formed within the developing caryopsis of wheat grew intercellularly within the confines of the epidermis and the integuments. Infection of the developing embryo was not observed.