COMPARATIVE ANATOMY AND HISTOCHEMISTRY OF THE ASSOCIATION OF *PUCCINIA POARUM* WITH ITS ALTERNATE HOSTS

By

TALIB OWAID AL-KHESRAJI

Department of Botany, University of Sheffield

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TO MY PARENTS

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Talib Owaid Al-Khesraji

Department of Botany, University of Sheffield

SUMMARY

The relationship of the macrocyclic rust fungus *Puccinia poarum* with its pycnial-aecial host, *Tussilago farfara*, and its uredial-telial host, *Poa pratensis*, has been investigated, using light microscopy, electron microscopy and micro-autoradiography. Aspects of the morphology and ontogeny of spores and sori, which were previously disputed, have been clarified.

Monokaryotic hyphae grow more densely in the intercellular spaces of *Tussilago* leaves than the dikaryotic intercellular hyphae on *Poa*. Although ultrastructurally similar, monokaryotic hyphae differ from dikaryotic hyphae in their interaction with host cell walls, often growing embedded in wall material which may project into the host cells.

The frequency of penetration of *Poa* mesophyll cells by haustoria of the dikaryon is greater than that of *Tussilago* cells by the relatively undifferentiated intracellular hyphae of the monokaryon. Intracellular hyphae differ from haustoria in their irregular growth, septation, lack of a neck-band or markedly constricted neck, the deposition of host wall-like material in the external matrix bounded by the invaginated host plasmalemma and in the association of callose reactions with intracellular hyphae and adjacent parts of host walls. Monokaryotic and dikaryotic infections differ also in the changes induced in the organization and ultrastructure of their respective host cells.

Intracellular hyphae in bundle sheath, xylem parenchyma, transfer cells of phloem parenchyma and companion cells, give the monokaryon of *P. poarum* direct access to nutrients translocated in vascular tissue of *Tussilago*. Bundle sheath cells of *Poa* contain unusually long haustoria but there is no penetration of the endodermis and vascular tissue by the dikaryon.

After uptake of tritiated glycerol by infected tissue, microautoradiographic investigation shows radioactivity to be concentrated in sporulation structures, haustoria and, in the case of *Poa*, in host nuclei. Cells of uninfected tissues differ from those of infected tissue in accumulation of label in chloroplasts. These studies contribute to an understanding of the physiological interaction of *P. poarum* with its alternate hosts.

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CHAPTER 1

INTRODUCTION

1

INTRODUCTION

Ecologically, rust fungi are obligate parasites of wild plants (Cummins 1959; Wilson & Henderson 1966) as well as cultivated crops, especially cereals (Johnson *et al.* 1967), although some have recently been grown axenically in pure culture or tissue culture (Coffey 1975). The relationship of rust fungi to their host plants shows the following features, which Dickinson & Lucas (1977) have listed as characteristic of biotrophic infections:

1. The host cells are not immediately killed; 2. Few or no toxins or extracellular enzymes are produced; 3. The fungi grow partially within the host cells by forming special intracellular structures; 4. Invasion of the host may occur directly or through stomata; 5. Narrow host range; 6. Dependence upon a living host to complete the rust life cycle; 7. The host may be infected at any stage of development.

The macrocyclic rust *Puccinia poarum*, which is readily maintained on its alternate hosts in growth-room conditions (McGee *et al.* 1973), has many advantages as a model system for physiological studies (Lewis 1976). Since large and discrete pycnial-aecial pustules are produced on leaves of *Tussilago farfara*, direct comparisons can be made between the healthy and infected tissue of the same leaf. The intracellular structures formed by *P. poarum* on *Tussilago* and on the alternate host, *Poa pratensis*, are morphologically different (Lösel & Lewis 1974; Lösel 1978). More detailed investigation of this feature and any

associated physiological differences may increase our understanding of such biotrophic parasites and their association with the higher plants.

Studies on the physiology (Holligan *et al.* 1973; Lösel & Lewis 1974; Long *et al.* 1975; Lewis 1976; Lösel 1978), development, cytology (Blackman & Fraser 1906) and ultrastructure of the aecial stage (Orvical 1968) and aeciospores (Henderson *et al.* 1972) of this rust have been reported. Orcival (1968) described ultrastructural changes in the chloroplast of *Tussilago farfara* during the development of the aecial infection by *P. poarum*, but no detailed, comparative investigation of the host-parasite interfaces and of changes in the cell organelles in both alternate hosts of this parasite has been previously reported. Such information is essential for a fuller understanding of the physiological relationship between the rust fungus and its host.

1.1 Morphology of sori and spores

The general features of *P. poarum* at the light microscope level were described by Grove (1913), Wilson & Henderson (1966) and Greene & Cummins (1967) but the occurrence of uredial and telial paraphyses, which are useful taxonomic features, requires more investigation. Plowright (1889) and Wilson & Henderson (1966) stated that paraphyses were not present in uredia of *P. poarum* while Greene & Cummins (1967) describe uredia as "mostly adaxial ..., usually without paraphyses but occasional specimens have short inconspicuous capitate, thin-walled paraphyses". The telial paraphyses of this rust were described

by Wilson & Henderson (1966) and Buchwald (1972), but Greene & Cummins (1967) found these structures variable in their development in telia of *P. poarum*.

Apart from the previous light microscope studies, other morphological features of spores and sori of *P. poarum*, such as flexuous hyphae, periphyses, germ pores (except of uredio-spores), peridia and the abundance of sori produced require investigation. No previous electron microscopic studies on the general features of this rust throughout its life cycle have been recorded.

The morphology and ontogeny of pycniospores (Rijkenberg & Truter 1974a; Harder & Chong 1978; Gold *et al.* 1979; Gold & Littlefield 1979), aeciospores (Moore & McAlear 1961; Rijkenberg & Truter 1974b; Gold *et al.* 1979; Gold & Littlefield 1979), urediospores (Littlefield 1971b; Harder 1976b; Hassan & Littlefield 1979) and teliospores (Harder 1977; Gold & Littlefield 1979) of other rusts have been studied using electron microscopy (see review by Littlefield & Heath 1979). Further investigation of rust spores and sori, using both scanning and transmission electron microscopy, may lead to a better understanding of their development and improved taxonomic characterization of rust species.

1.2 Vegetative growth of rust fungi

1.2.1 Intercellular hyphae

The growth and structure of the intercellular mycelium of rust fungi and its relationship to host tissues have received much less attention than the intracellular structures and sporulating stages.

a) Cell wall. The hyphal cell wall in rust fungi appears to be composed of two fibrillar layers and an additional outer amorphous layer, which can be recognized in both monokaryotic (Boyer & Isaac 1964; Rijkenberg & Truter 1973; Robb *et al.* 1973; Walles 1974; Welch & Martin 1975) and dikaryotic hyphae (Rijkenberg & Truter 1973; Muller *et al.* 1974; Rijo & Sargent 1974). It has been suggested that the amorphous outer layer may be adhesive in nature (Hardwick *et al.* 1971; Rijkenberg & Truter 1973; Muller *et al.* 1974; Walles 1974) or may have a protective property against host toxins (Boyer & Isaac 1964).

As in other basidiomycetes, intercellular hyphae of rust fungi are septate. However, at the electron microscope level, three basic types of septum are recognized in the Uredinales (Littlefield 1971a; Littlefield & Bracker 1971; Littlefield and Heath 1979): (i) perforate septa; (ii) complete septa and (iii) partial septa.

(i) The perforate septum, generally considered to be the typical rust septum, develops centripetally (Littlefield & Bracker 1971; Rijkenberg & Truter 1974b; Harder 1976a). It consists of cross-wall, septal pore and the organelle-free septal pore apparatus (Bracker 1967; Ehrlich *et al.* 1968; Littlefield & Bracker 1971; Jones 1973; Walles 1974). The septal pore is usually less than 100 nm and may be much less than that (*Melampsora lini*, Littlefield & Bracker 1971). Characteristically, vesicles (Ehrlich *et al.* 1968; Jones 1973) and microbodies (Littlefield & Bracker 1971; Coffey *et al.* 1972a; Robb *et al.* 1973; Muller *et al.* 1974; Rijo & Sargent 1974; Walles 1974) are found at the periphery of the pore apparatus. The presence of an electron-dense pore plug in

the septalpore in hyphae of rust fungi has been reported by many workers (Longo & Naldini 1970; Littlefield & Bracker 1971; Jones 1973; Robb *et al.* 1973; Walles 1974; Harder 1976a; Mims & Glidewell 1978). Jones (1973) believed that the pore plug originates from the electron-dense peripheral vesicles. Walles (1974) suggested that it represented woronin bodies which frequently occur in Ascomycetes (Bracker 1967; Wergin 1973). Physiologically, Ehrlich *et al.* (1968) suggested that the septal pore might maintain the uninucleate or binucleate condition in each cell.

ii) Complete septa, lacking a pore and pore apparatus, are commonly seen in pseudoparenchyma of uredial and aecial primordia (Moore 1963a; Littlefield & Bracker 1971).

iii) Partial septa (Littlefield & Bracker 1971), pseudosepta (Ehrlich *et al.* 1968) or infolding walls (Rijkenberg & Truter 1975) differ in their morphology and development from the previous types, in that they seem to be formed by the infolding of the longitudinal walls of the hyphae. No pore apparatus and pore plug is present in this type of septum. Partial septa are found in primordia of aecia, uredia, and telia (Moore 1963a; Littlefield & Bracker 1971; Muller *et al.* 1974; Rijkenberg & Truter 1974b; Mims & Glidewell 1978) and in other regions of the infected tissue (Ehrlich *et al.* 1968; Muller *et al.* 1974). Manocha & Shaw (1967) reported that partial septa occur in germ tubes of *Melampsora lini*. Apart from the studies of Moore (1963a) and Walles (1974), further ultrastructural investigation of septa associated with the pycnial and aecial stages of rust

fungi is needed. The correlation between septal structures and nuclear migration was discussed by Giesy & Day (1965).

b) Cell contents. Although the ultrastructure of the intercellular hyphae of rust fungi has not been extensively studied, the available evidence suggests that their protoplasm generally resembles that of the septate intercellular hyphae of parasitic or non-parasitic fungi (Bracker 1967; Littlefield & Heath 1979). Two nuclei per cell are observed in dikaryotic hyphae (Manocha & Shaw 1967; Coffey *et al.* 1972a; Muller *et al.* 1974; Rijo & Sargent 1974) while in monokaryotic mycelium only one nucleus per cell is seen (Walles 1974). The mitochondria are associated with cisternae of endoplasmic reticulum (Muller *et al.* 1974; Coffey 1975). Coffey *et al.* (1972a) reported that the number of cristae in mitochondria of the intercellular hyphae of both *Puccinia helianthi* and *Melampsora lini* was less than that in haustoria. Microbodies were located either at the periphery of the pore apparatus, as in perforate septa, or dispersed in the cytoplasm (Coffey *et al.* 1972a; Heath & Heath 1975). Unlike the microbodies observed in the infection structures (Mendgen 1973b; Littlefield & Heath 1979), those in the vegetative mycelium sometimes contain a crystal (Coffey *et al.* 1972a). Vesicles with membranous material and vacuoles are frequently seen in intercellular hyphae of rust fungi (Walles 1974; Littlefield & Heath 1979). Microtubles have been reported only in intercellular hyphae of *Puccinia helianthi* (Coffey *et al.* 1972a), *Melampsora lini* (Coffey 1975) and *Uromyces phaseoli* var. *vignae* (Heath & Heath 1978). The only storage materials observed in intercellular mycelium are lipid droplets and glycogen particles (Van Dyke & Hooker 1969; Zimmer 1970; Coffey *et al.* 1972a;

Walles 1974; Coffey 1975; Harder 1978). In addition to these structures, endoplasmic, reticulum, ribosomes and lomasomes (e.g. Van Dyke & Hooker 1969; Zimmer 1970; Walles 1974) are found in intercellular hyphae of rust fungi. Golgi bodies have not been detected in the Uredinales, although tubular or membranous structures in intercellular hyphae of certain rust species have been interpreted as Golgi bodies (Moore 1963b; Pinon *et al.* 1972) or as structures resembling Golgi bodies in their function (Rijo & Sargent 1974).

1.2.2 Fungal intracellular structures in host cells

Like many other biotrophic fungi such as powdery mildews and downy mildews (Bushnell 1972; Bracker & Littlefield 1973), the intercellular hyphae of rusts produce special branches penetrating the host cell walls, which appear to serve as the principal nutritional interface between the autotrophic host and fungal pathogen. These branches are referred to as intracellular structures, although the host plasma membrane remains unpenetrated. Most studies of intracellular structures produced by rust fungi have been confined to the dikaryotic phase on uredial-telial hosts. Apart from the early light microscope observations (Colley 1918; Rice 1927; Allen 1935; Buller 1950), relatively little attention has been paid in recent years to the host-parasite interfaces of pycnial-aecial stages of rust infections (Rijkenberg & Truter 1973; Walles 1974; Harder 1978; Gold *et al.* 1979; Gold & Littlefield 1979).

In her classical study, Rice (1927) reported that the haustoria of uredial and telial phases of rusts differ from the intercellular structures of pycnial and aecial stages, which Allen (1935)

termed intracellular hyphae. The intracellular structures of macrocylic rusts throughout both monokaryotic and dikaryotic phases of the life cycle have been investigated at the electron microscope level only by Harder (1978) and Gold & Littlefield (1979). Some light microscope (Rice 1927; Lösel & Lewis 1974) and electron microscope (Moore & McAlear 1961; Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978; Gold *et al.* 1979) studies indicate that typical haustoria are absent in pycnial and aecial stages of rust infections, although Robb *et al.* (1975b) considered the haustoria in pycnial-aecial stages of *Cronartium ribicola* on tissue culturesof *Pinus monticola* as intermediate between typical uredial haustoria and intracellular hyphae but, like previous workers, found their occurrence sparse in relation to the extensive growth of the intercellular mycelium. In the present work, the term "intracellular hypha" will be used for the fungal structures produced within host cells in pycnial-aecial infection.

a) Haustoria. Studies with light microscope (Rice 1927, Littlefield 1972; Prusky *et al.* 1980) and scanning electron microscope (Pring & Richmond 1975; Gold *et al.* 1979; Plotnikova *et al.* 1979) show that the typical dikaryotic haustoria are differentiated into an expanded body and narrow, tubular neck. The haustorial neck is attached to the haustorium mother cell, which is a terminal cell of the intercellular mycelium. The thickening of the haustorium-mother cell wall at the point of penetration has been reported by many workers (e.g. Coffey *et al.* 1972a; Rijkenberg & Truter 1973; Rijo & Sargent 1974; Harder 1978; Borland & Mims 1980).

The fungal cytoplasm is continuous between the haustorium-mother cell and haustorial body (Coffey *et al.* 1972a) with no intervening septum. The contents of the haustorium-mother cell have been reported to migrate into the haustorium (Heath & Heath 1971, 1975; Mendgen 1975). Vacuolation of the haustorium-mother cell accompanies this process (Coffey 1976).

The haustorial neck is wider than the penetration peg and extends for some distance into the host cell lumen. The neckband (or neck ring) is located about halfway along the haustorial neck. Generally, neckbands have been observed in all rust haustoria examined (Van Dyke & Hooker 1969; Hardwick *et al.* 1971; Rijkenberg & Truter 1973; Coffey 1976; Harder 1978; Borland & Mims 1980). Bossanyi & Olah (1974) suggested that the neckband in *Puccinia graminis* f.s. *tritici* marks a site of exchange of material between the fungus and its host. The neckband of *Melampsora lini* appears to support this suggestion (Coffey *et al.* 1972a; Littlefield & Bracker 1972; Coffey 1976). The haustorial body is formed by the expansion of the distal end of the haustorial neck. Variations in haustorial morphology have been reported in several rust studies (Rice 1927; Rajenderen 1972).

The fungal cytoplasm is separated from the host cytoplasm by a complex region comprising the fungal plasma membrane, the fungal wall, and the host plasma membrane. An additional layer is usually located between the haustorial wall and the host plasma membrane. Different terminology has been used to describe this layer in various studies of obligate parasites (Ehrlich & Ehrlich 1971; Bushnell 1972; Littlefield & Heath 1979). It has been termed a sheath (Smith 1900;

Fraymouth 1956; Ehrlich & Ehrlich 1963b; Bracker 1968; Chou 1970; Zimmer 1970; Littlefield & Bracker 1972; Rijkenberg & Truter 1973; Coffey 1975), a sack (Hirata & Kojima 1962), a zone of apposition (Peyton & Bowen 1963), an encapsulation (Ehrlich & Ehrlich 1963a, 1966, 1971), extra-haustorial matrix (Bushnell 1972; Coffey 1976; Hickey & Coffey 1977; Littlefield & Heath 1979) and extra-haustorial sheath (Harder 1978).

The origin, nature or composition and the function of the matrix surrounding haustoria of the obligate parasites are still speculative and this leads to confusion in terminology (see Figure 1.1), in addition to the misuse of certain terms referring to different structures (i.e. sheath used differently by Bracker (1968) and Bushnell (1972)). References to the extensive literature concerning the origin of the matrix, the nature of the material which it contains and its possible function are summarized in Tables 1.1, 1.2 and 1.3.

It has been reported that the thickness (Shaw & Manocha 1965b; Manocha & Shaw 1967; Orcival 1969; Bushnell 1972; Harder 1978) and the amount of electron-dense material (Shaw & Manocha 1965b; Manocha & Shaw 1967; Orcival 1969; Zimmer 1970; Coffey *et al.* 1972a; Manocha 1975; Harder 1978) of the extrahaustorial matrix increase with the age of the haustorium. However, Kajiwara (1971) found a thick extrahaustorial matrix around a young haustorium.

In some studies, it has been claimed that the production of extrahaustorial matrix in resistant and susceptible hosts was different. Manocha (1966) reported a more rapid development of this matrix around haustoria in a resistant than in a susceptible host.

Table 1.1 Origin of matrix* (or comparable region) in biotrophic fungal infections.

Host in origin			Fungal in origin			Product of host-parasite interaction		
Author and data of publication	Host	Parasite	Author and date of publication	Host	Parasite	Author and date of publication	Host	Parasite
Smith 1900	*Geranium maculatum*	*Erysiphe communis*	Caporali 1960	*Rosa pouzine*	*Sphaerotheca pannosa*	McKeen *et al.* 1966	*Helianthus annuus*	*Erysiphe cichoracearum*
Hirata 1958	Barley	Powdery mildew	Ehrlich & Ehrlich 1963a	Wheat	*Puccinia graminis tritici*			
Kohima & Hirata 1961	Barley	Powdery mildew	Boyer & Isaac 1964	*Pinus strobus*	*Cronartium ribicola*	Zimmer 1970	Safflower	*Puccinia carthami*
Peyton & Bowen 1963	*Glycine max*	*Peronospora manshurica*	Bracker 1968	Barley	*Erysiphe graminis*			
Berlin & Bowen 1964	*Raphanus sativus*	*Albugo candida*	Chou 1970	Cabbage	*Peronospora parasitica*			
Shaw & Manocha 1965b	Wheat	*Puccinia graminis tritici*	Robb *et al.* 1975b	*Pinus monticola*	*Cronartium ribicola*			
Calonge 1969	*Hordeum vulgare*	*Puccinia hordei*	Mims & Glidewell 1978	*Juniperus virginiana*	*Gymnosporangium juniperi-virginianae* Schw.			
Hardwick *et al.* 1971	*Phaseolus vulgaris*	*Uromyces appendiculatus*						
Heath 1971	Cowpea	*Uromyces phaseoli vignae*						
Manocha 1975	Wheat	*Puccinia graminis tritici*	Glidewell & Mims 1979	*Robus trivialis* Mich x	*Kunkelia nitens*			
Mendgen & Heitefuss 1975	*Phaseolus vulgaris*	*Uromyces phaseoli*						
Pring & Richmond 1976	*Phaseolus vulgaris*	*Uromyces phaseoli*						
Harder 1978	*Avena sativa*	*Puccinia coronata*						

*For definition of matrix see Fig.1.1.

Table 1.2 Composition of matrix* in biotrophic fungi parasitic on higher plants.

Author and date of publication	Matrix Nature or composition	Host	Parasite
Caporali 1960	Pectic substances	*Rosa pouzine*	*Sphaerotheca pannosa*
Ehrlich & Ehrlich 1963a	Particulate	Wheat	*Puccinia graminis tritici*
Peyton & Bowen 1963	Stains darkly but different from host cell wall	*Glycine max*	*Peronospora manshurica*
Berlin & Bowen 1964	Amorphous and moderately electron-dense	*Raphanus sativus*	*Albugo candida*
Shaw & Manocha 1965b	Granular, electron-dense	Wheat	*Puccinia graminis tritici*
Bracker 1968	Amorphous and flexible	Barley	*Erysiphe graminis*
Calonge 1969	Granular	*Hordeum vulgare*	*Puccinia hordi*
Van Dyke & Hooker 1969	Amorphous with electron-dense bodies	*Zea mays*	*Puccinia sorghi*
Zimmer 1970	Fluid property	Safflower	*Puccinia carthami*
Hardwick *et al.* 1971	Callose and other fibrillar material	*Phaseolus vulgaris*	*Uromyces appendiculatus*
Littlefield & Bracker 1972	Electron-transparent with granular and electron-dense material	Flax	*Melampsora lini*
Rijo & Sargent 1974	Fibrillar	*Coffea arabica*	*Hemileia vastatrix*
Walles 1974	Electron-transparent with electron-dense particles	*Pinus sylvestris*	*Peridermium pini*
Manocha 1975	Electron-transparent with particulate material	Wheat	*Puccinia graminis tritici*
Coffey 1976	Electron-lucent with fibrillar material	Flax	*Melampsora lini*
Perera & Gay 1976	Granular	Rose	*Sphaerotheca pannosa*
Harder 1978	Electron-lucent with fibrillar material	*Avena sativa*	*Puccinia coronata avenae*
Hickey & Coffey 1978	Electron-dense layer of proteinaceous and carbohydrate compounds	*Pisum sativum*	*Peronospora pisi*
Mims & Glidewell 1978	Granular and stains similarly to haustorial wall	*Juniperus virginiana*	*Gymnosporangium juniperi-virginiana*
Glidewell & Mims 1979	Stains similarly to haustorial wall but more electron-dense	*Rubus trivialis*	*Kunkelia nitens*

*For definition of matrix see Fig.1.1.

Table 1.3 Function of matrix* in biotrophic fungi parasitic on higher plants

Author and date of publication	Function of matrix
Ehrlich & Ehrlich 1963a,b; Peyton & Bowen 1963; Berlin & Bowen 1964; Shaw & Manocha 1965b; Ehrlich *et al.* 1966; Zimmer 1970; Favali & Marte 1973; Rijo & Sargent 1974; Coffey 1975	Transfer of substances between host and parasite
Rice 1927; Shaw & Manocha 1965b; Manocha 1966, 1975; Manocha & Lee 1972; Coffey 1976; Manocha & Letourneau 1978	Associated with host resistance
Calonge 1969; Hardwick *et al.* 1971; Hickey & Coffey 1977	May be laid down by the host to prevent the development of haustoria or to isolate haustoria
Davison 1968; Manocha & Lee 1971	May reduce or limit metabolic interchange between host and parasite
Heath 1971	Could be a host response to the toxic effect of the haustorium
Robb *et al.* 1975b	Effective shield against host toxic material
Ehrlich & Ehrlich 1971	Buffer against toxic effect of one entity upon the other
McKeen *et al.* 1966	Serve no useful purpose

*For definition of matrix see Fig.1.1

Zimmer (1970) mentioned that the extrahaustorial matrix appeared similar in both resistant and susceptible hosts but later it appeared more electron-dense in susceptible than in resistant hosts. However, Van Dyke & Hooker (1969) found no differences in the extrahaustorial matrix in resistant and susceptible hosts.

The boundary of the extrahaustorial matrix (or comparable structures) is agreed to be the invaginated host plasma membrane (Thatcher 1943; Fraymouth 1956; Bracker 1968; Bushnell 1972). Some recent studies indicate that the staining properties (Littlefield & Bracker 1970, 1972), the thickness (Ehrlich & Ehrlich 1963a; Bracker 1968) and the function (Ehrlich & Ehrlich 1971) of the extrahaustorial membrane (invaginated plasma membrane) are different from that of the non-invaginated host plasma membrane.

The extrahaustorial membrane may be involved in several important activities. Ehrlich & Ehrlich (1971) and Littlefield & Bracker (1972) suggested that it has a special role in the response of a host to infection. Other workers have indicated that the extrahaustorial membrane may be engaged in the movement of materials from the host cytoplasm to the haustorium or *vice versa* (reviews by Bracker 1968; Ehrlich & Ehrlich 1971; Littlefield & Heath 1979). Observations supporting this include the invagination of the extrahaustorial membrane (Ehrlich & Ehrlich 1963b; Bracker 1967; Van Dyke & Hooker 1969), the continuity with tubules of the host (Bracker & Littlefield 1973; Harder *et al.*1978), the pinocytotic-like vesicles (Ehrlich & Ehrlich 1963a; Van Dyke & Hooker 1969) and the association with the endoplasmic reticulum of the host (reviews by Bracker 1967; Littlefield & Heath 1979).

Generally, similar cytoplasmic components are present in both intercellular hyphae and haustoria. Haustoria are usually reported to have two nuclei (Rijkenberg & Truter 1973; Abu-Zinada *et al.* 1975; Coffey 1976) but several workers have observed only one nucleus in the haustorial body (Zimmer 1970; Hardwick *et al.* 1971; Mims & Glidewell 1978). Mitochondria (e.g. Zimmer 1970; Coffey *et al.* 1972a; Abu-Zinada *et al.* 1975; Coffey 1976), endoplasmic reticulum (Zimmer 1970; Littlefield & Bracker 1972), ribosomes (Zimmer 1970; Hardwick *et al.* 1971; Littlefield & Bracker 1972), lomasomes (Van Dyke & Hooker 1969; Zimmer 1970) and multivesicular bodies (Hardwick *et al.* 1971; Abu-Zinada *et al.* 1975) are also observed in haustoria of rust fungi. Moreover, microtubules (Hardwick *et al.* 1971; Coffey *et al.* 1972a), vacuoles (e.g. Hardwick *et al.* 1971; Rijo & Sargent 1974; Abu-Zinada *et al.* 1975), lipid droplets (Van Dyke & Hooker 1969; Coffey *et al.* 1972a; Rijo & Sargent 1974) and glycogen (Van Dyke & Hooker 1969; Zimmer 1970; Coffey *et al.* 1972a) have also been found in haustoria. Microbodies have, however, rarely been seen in haustoria of rust fungi (Mendgen 1973a).

b. Intracellular hyphae. Several light microscopic (Colley 1918; Rice 1927; Allen 1932; Lösel & Lewis 1974) and electron microscopic (Moore & McAlear 1961; Boyer & Isaac 1964; Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978; Gold *et al.* 1979; Borland & Mims 1980) studies reveal that the pycnial and aecial stages of rust fungi produce intracellular structures designated P-haustoria by Harder (1978), M-haustoria by Littlefield & Heath (1979) and intracellular hyphae by Allen (1936) and Gold *et al.*

(1979). These intracellular structures have been described as hypha-like or filamentous (Rice 1927; Allen 1932; Rijkenberg & Truter 1973; Lösel & Lewis 1974; Gold *et al.* 1979) and septate (Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978; Gold & Littlefield 1979). Gold & Littlefield (1979) mention that the intracellular hyphae were observed to emerge from one cell to another, while most other studies found the intracellular hyphae restricted to one host cell (Walles 1974; Robb *et al.* 1975b; Harder 1978; Borland & Mims 1980). The typical haustorial neck and neck-band have not been seen in intracellular hyphae (Rijkenberg & Truter 1973; Harder 1978; Gold *et al.*1979) although Robb *et al.* (1975b) reported neckband-like structures in intracellular hyphae of *Cronartium ribicola* grown in tissue culture. It has been assumed that these neckband-like structures are artifacts associated with fungal necrosis (Littlefield & Heath 1979). The fungal wall appears continuous between the mother cell and the intracellular hypha. No localized thickening of the mother cell wall at the point of entry to the host cell has been seen (Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978; Gold *et al.* 1979). The intracellular hypha-mother cell has been reported to be a terminal cell, separated from the rest of the mycelium by a septum (Harder 1978). In contrast, other light microscopic observations indicate that the intracellular hypha-mother cell need not be terminal (Colley 1918; Allen 1935).

The cytoplasmic contents of the intracellular hyphae resemble those of the uredial and telial haustoria (Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978; Gold *et al.* 1979).

Harder (1978) mentioned that glycogen occurred in intracellular hyphae (P-haustoria) of *Puccinia coronata avenae* but not in the uredial haustoria of the parasite. Variations occur in the number of nuclei in intracellular hyphae of several rusts (Rijkenberg & Truter 1973; Robb *et al.* 1975b; Kohno *et al.* 1976, 1977; Borland & Mims 1980). However, there appears to be no information on the migration of the cytoplasm from the mother cell to the intracellular hyphae (Littlefield & Heath 1979). Similarly to uredial haustoria, intracellular hyphae are enclosed by the invaginated host plasma membrane (Rijkenberg & Truter 1973; Walles 1974; Robb *et al.* 1975b; Harder 1978) and a matrix of unknown origin, nature and function between the fungal wall and this membrane (see review by Littlefield & Heath 1979).

Littlefield & Heath (1979) pointed to the need of more detailed studies to answer several questions concerning the intracellular hyphae, such as their mode of formation, the presence or absence of some structural and morphological features in intracellular hyphae and the host-parasite interface.

The general terminology used by the previous studies (Peyton & Bowen 1963; Berlin & Bowen 1964; Calonge 1969; Bushnell 1972; Coffey 1976; Hickey & Coffey 1977; Littlefield & Heath 1979) is shown in Figure 1.1, which is a diagramatic representation of the fungal and host structures associated with the intracellular organs of the obligate parasites. The collar (P+S) region is thought to be an extension of the host cell wall (Peyton & Bowen 1963; Berlin & Bowen 1964; Shaw & Manocha 1965b; Ehrlich & Ehrlich 1966; Manocha & Shaw 1967). In powdery mildews, Luke *et al.* (1966), Edwards &

Allen (1970) and Hanchey & Wheeler (1971) suggested that a papilla forms as a result of host cytoplasmic activity. Bushnell (1972) reported that papilla (P) and sheath (S) were distinct from the host cell wall. However, these studies suggest that the collar (P+S) is deposited by the host as well as the encasement (Ehrlich & Ehrlich 1971; Littlefield & Bracker 1972; Pring & Richmond 1976; Hickey & Coffey 1977). Other studies suggest that the production of sheath material around the necks of haustoria is induced by substances (probably enzymes) secreted by the fungus (McKeen 1969; Leong *et al.* 1970).

In Figure 1.1, the intracellular structure and its surrounding membrane (PL) is enclosed by the encasement (E) which is surrounded by the host plasma membrane (HPL) (Littlefield & Bracker 1972; Pring & Richmond 1976; Hickey & Coffey 1977). Vesicular and tubular profiles are seen in the encasement (Ehrlich & Ehrlich 1971; Coffey 1975, 1976; Littlefield & Heath 1979).

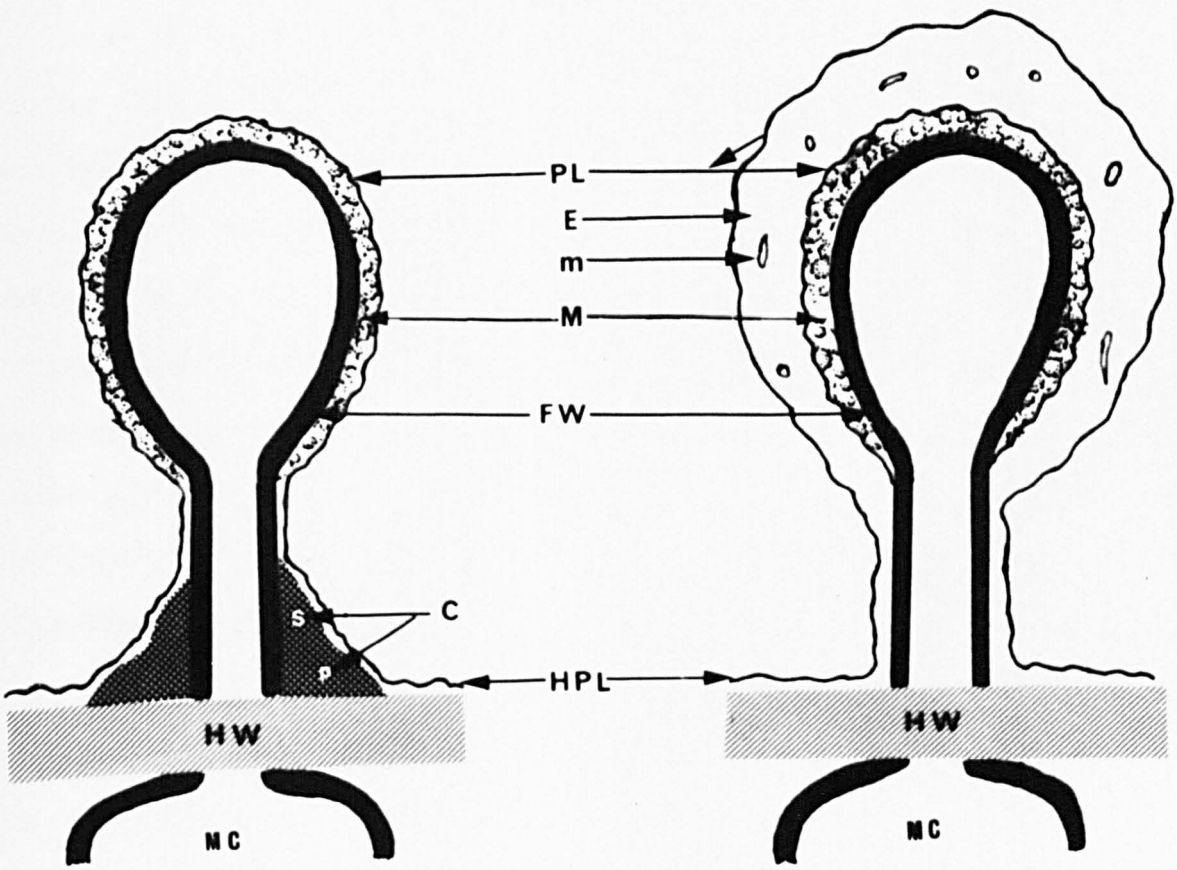
The composition of the collar in host-parasite combination is unknown, although it was reported to be composed of callose (Fraymouth 1956; Davison 1968; Edwards & Allen 1970; Hardwick *et al.* 1971; Heath & Heath 1971) or cellulose (Colley 1918). However, cellulose or callose was not detected in sheaths around the necks of haustoria of *Erysiphe graminis* (Bracker 1968).

Figure 1.1

Diagramatic representation of the terminology used by various workers (pp.9 & 10) to describe the fungal and host structures associated with the intracellular organs of obligate parasites.

P, papilla; S, sheath; C, collar; E, encasement; M, matrix between fungal wall and the invaginated part of the host plasma membrane (i.e. extrahaustorial matrix or other comparable terms)

HPL, non-invaginated host plasma membrane; PL, invaginated host plasma membrane; FW, fungal wall; HW, host wall; MC, mother cell of the intracellular structure; m, membranous material in the encasement.


PL
E
m
M
FW
C
S
P
HPL
HW
MC
HW
MC

1.3 Host responses

1.3.1 Sheath, encasement and other changes associated with host cell wall

As indicated by Ehrlich & Ehrlich (1971) and Littlefield & Bracker (1972), the involvement of the host plasma membrane in the host response to infection has been reported in various plant diseases other than rusts (Wheeler & Hanchey 1968; Hess & Strobel 1970; Strobel & Mathre 1970; Goodman & Plurad 1971). In response to the pathogen, host wall-like material forms in advance of the penetrating parasite (Hardwick *et al.* 1971; Bracker & Littlefield 1973; Walles 1974) or after the development of haustoria inside the host cell (Heath & Heath 1971; Coffey *et al.* 1972; Littlefield & Bracker 1972; Mims & Glidewell 1978). Littlefield & Bracker (1972) considered that the encasement was deposited by the host cell wall. Heath & Heath (1971) suggested that the encasement was associated with the resistance of certain host-parasite combinations, but that it might represent a non-specific host response after penetration by the parasite (Coffey 1976). Pring & Richmond (1976) reported that treatment with the fungicide, oxycarboxin, induced the formation of the encasement around haustoria of *Uromyces phaseoli*. Similar deposition was produced by inserting a glass microprobe into a living tobacco plant cell (Nims *et al.* 1967). Encasement formation has been reviewed by Ehrlich & Ehrlich (1971), Bushnell (1972) and Littlefield & Heath (1979).

In addition to the formation of papilla, sheath and encasement, other changes associated with the host cell wall have been reported in rust-infected plants. Thickening of host cell walls between infected and uninfected cells was noted in infections by *Puccinia graminis* and *Puccinia triticina* on wheat plants (Allen 1923, 1927 reviewed by Reynolds 1974). Similarly Heath (1972), using the electron microscope, found that electron opaque material was deposited on and within the host cell walls of the non-host *Phaseolus vulgaris* in response to infection hyphae of cowpea rust.

1.3.2 Changes in host cell organelles

a) Endoplasmic reticulum and Golgi bodies. The rust infection of higher plants is characterized by producing remarkable changes in the host organelles. The intracellular structures of both monokaryon and dikaryon are often observed in close association with the host endoplasmic reticulum (Van Dyke & Hooker 1969; Zimmer 1970; Ehrlich & Ehrlich 1971; Heath & Heath 1971; Heath 1972; Littlefield & Bracker 1972; Abu-Zinada *et al.* 1975; Harder 1978). Connections between endoplasmic reticulum and the extrahaustorial membrane have been reported by Harder *et al.* (1978). This is assumed to be important in facilitating the movement of material from the host to the parasite (Littlefield & Heath 1979). Rough endoplasmic reticulum has also been observed around the haustorium (Hardwick *et al.* 1971; Heath & Heath 1971; Littlefield & Heath 1979). Heath & Heath (1971) suggested that extrahaustorial membrane might arise from vesicles produced by rough endoplasmic reticulum. An increase in endoplasmic reticulum and

the number and activity of Golgi bodies in response to infection has been detected in the invaded host cells (Shaw & Manocha 1965b; Manocha & Shaw 1967; Van Dyke & Hooker 1969; Ehrlich & Ehrlich 1971; Robb *et al.* 1975b). Relatively few studies of rust infection have reported the presence of cytoplasmic vesicles in the vicinity of the haustorium (Ehrlich & Ehrlich 1963a; Shaw & Manocha 1965b; Manocha & Shaw 1967; Abu-Zinada *et al.* 1975). Ehrlich & Ehrlich (1971) found little evidence to support the theory that Golgi-derived vesicles were involved in the transport of material from or to the haustorium.

b) Chloroplasts. The chloroplasts of infected host cells are often closely associated with uredial haustoria (Van Dyke & Hooker 1969; Coffey *et al.* 1972a,b; Coffey 1976). Such association has not been reported for the pycnial infections although some micrographs of intracellular hyphae show the close proximity of host chloroplasts (Littlefield & Heath 1979). In rust-infected sunflower, Sood & Sackston (1970) found fewer chloroplasts in infected than uninfected mesophyll cells. Similarly, Mares (1979) reported that the host cells in the chlorotic infected tissue contained reduced numbers of chloroplasts. However, both studies showed no statistical evidence. A decrease in size of the chloroplasts in the infected zones was previously reported (Rothman 1960, *Puccinia coronata* on oat; Whitney *et al.* 1962, *Puccinia triticina* on wheat; Hilu 1965, *Puccinic sorghi* on *Zea mays*.).

Ultrastructural changes in the chloroplasts of rust-infected tissue have been primarily studied in uredial and telial stages

(Thomas & Isaac 1967; Coffey *et al.* 1972b; Heath 1974; Abu-Zinada *et al.* 1975; Mlodzianowski & Siwecki 1975). These changes included increased number and/or size of osmiophilic globules (Thomas & Isaac 1967; Coffey *et al.* 1972b; Heath 1974; Abu-Zinada *et al.* 1975; Mlodzianowski & Siwecki 1975), increased formation of peripheral reticulum (Coffey *et al.* 1972b; Heath 1974; Abu-Zinada *et al.* 1975), the reduction in the number of photosynthetic lamellae (Abu-Zinada *et al.* 1975) and the disruption of the membrane system (grana and intergranal lamellae) (Coffey *et al.* 1972b; Heath 1974; Mlodzianowski & Siwecki 1975; Mares 1979). Chromoplast-like changes, particularly the formation of the prolamellar bodies, were reported by Orcival (1968) in pycnial-aecial stages of infection of *Tussilago farfara* by *Puccinia poarum*, and also by Coffey *et al.* (1972b) and Heath (1974). It was demonstrated that ethylene gas could be responsible for the development of chromoplast-like chlotoplasts (Heath 1974). Daly *et al.* (1972) and Heath (1974) reported that the production of ethylene gas was increased during rust infection. The role of this gas in regulating fruit ripening (Burg & Burg 1962, 1965), senescence (Burg 1968; Abeles 1973) and abscission (Burg 1968) was studied previously.

Starch degradation within chloroplasts in rust infection has been observed by Coffey *et al.* (1972) and Mlodzianowski & Siwecki (1975), while Heath's study (1974) showed numerous undegraded starch grains within the chloroplast of infected cowpea leaves. Several workers (Shaw & Manocha 1965a, b; Coffey *et al.* 1972b; Orcival 1972) reported that the ultrastructural changes in the chloroplasts of rust-infected tissue are generally similar to those observed in natural leaf senescence (Ikeda & Ueda 1964; Shaw & Manocha 1965a;

Ljubesic 1968). In contrast other rust studies (Orcival 1968; Coffey *et al.* 1972b; Heath 1974) indicated that these alterations closely resemble chromoplast development in ripening fruit (Thompson 1966; Spurr & Harris 1968). However, similar changes in the chloroplast have been described in various plants infected by viruses (Esau 1968; Matthews 1973; Tomlinson & Webb 1978) or bacteria (Lallyett 1977) and during chemical treatments (Burg 1968; Butler & Simon 1968; Fischer *et al.* 1973; Heath 1974).

c) Mitochondria and microbodies. Only one detailed study (Coffey *et al.* 1972b) has reported alterations in the mitochondria and microbodies of rust-infected tissue. Coffey *et al.* (1972b) showed that in sunflower infected by *Puccinia helianthi* but not in flax infected by *Melampsora lini*, the mitochondria contain atypical plate-like cristae and occasional crystals while in tissues infected by *Puccinia helianthi* and *Melampsora lini*, the microbodies frequently contain crystalline cores.

d) Nucleus. The close association of the host nucleus and uredial haustoria (Rice 1927; Allen 1928; Zimmer 1965; Manocha & Shaw 1966; Shaw 1967; Van Dyke & Hooker 1969; Heath & Heath 1971; Coffey *et al.* 1972b; Coffey 1975) or intracellular hyphae of pycnial infections (Rijkenberg & Truter 1973; Robb *et al.* 1975a; Gold *et al.* 1979) has been suggested to be the characteristic host response to invasion (Littlefield & Heath 1979).

Increase in size of nuclei in cells of both suscepible and resistant hosts was previously reported (Allen 1926, 1927; Sood & Sackston 1970). Sood & Sackston (1970) found that the size of nuclei of

a susceptible host (sunflower) increased much more than that of the resistant host. They concluded that the increase in nuclear size was related to susceptibility.

Changes in the ultrastructure of the host nucleus have been studied in some detail during uredial infection where the density of the interchromatin material was reported to be increased (Manocha & Shaw 1966; Abu-Zinada *et al.* 1975). Robb *et al.* (1975a) mentioned that the heterochromatin was notably decreased in nuclei of cultured pine cells infected by *Cronartium ribicola*. However, the increase in nuclear RNA and decrease in nuclear DNA suggested a specific action of the parasite on the host metabolism (Whitney *et al.* 1962). No comparative reports on the host nucleus ultrastructure during pycnial and uredial stages of a single rust are available.

1.4 Relationship of rust fungi to host vascular bundle

There is little information available concerning the association of rust fungi with vascular bundles of their hosts. In his classical study, Colley (1918) described the penetration of the phloem, cambium, medullary ray tissue and even tracheids of pine branches by haustoria of pycnial-aecial infections of *Cronartium ribicola*. Jackson & Parker (1958) reported the invasion of the parenchymatous cells associated with the phloem and woody rays of loblolly pine by haustoria of *Cronartium fusiforme*. More recently, Van der Kamp (1969) and Walles (1974) found intracellular structures of *Peridermium (Cronartium) pini* in the vascular bundles of the stem of woody hosts. Walles (1974) reported that haustoria of the monokaryon occur in

parenchymatous parts of the vascular bundle, in young sieve cells, and in the tracheids of the outermost annual ring, although the mycelium was mainly in the cortex. Following infection of needles of *Pinus pini* by the endocyclic rust, *Peridermium pini* (Pers) Lev. Van der Kemp (1970) noted growth of fungal hyphae down the length of the leaf vascular bundle to intiate stem lesions.

Apart from Zimmer's finding (1965) of gametophytic hyphae of *Puccinia carthami* in protoxylem and metaxylem vessels of safflower seedling hypocotyls and Harder's (1978) electron micrographs showing fungal structures in vascular tissues of the pycnial host of *Puccinia coronata*, little attention has been paid to the fungal penetration of the vascular bundle of herbaceous hosts. However, no reports of inter- and intra-cellular structures of the dikaryon infecting vascular tissue of the host have been encountered. Gold *et al.* (1979), in their investigation of the haploid thallus of *Puccinia recondita* found no fungal penetration of the host vascular bundle.

1.5 Autoradiographic studies

Several studies on rusts and other obligate plant parasites have demonstrated an accumulation of various substances at the site of infection. Shaw & Samborski (1956) reported the accumulation of radioactive substances (^{14}C, ^{32}P, ^{45}Ca) in leaves at the uredial and conidial colonies of *Puccinia* and *Erysiphe* respectively. In wheat leaves infected with *Puccinia recondita* and fed with tritiated cytidine, the host cells in infected leaf areas contained less label

in their nuclei and cytoplasm than cells from the uninfected areas (Nielsen & Rohringer 1963). In contrast, Bhattacharya & Shaw (1967) found that the mesophyll cells in infected zones incorporated more leucine -^{3}H into protein and more cytidine -^{3}H and uridine -^{3}H into RNA than those of the uninfected areas of the same leaf after feeding tritium-labelled cytidine, leucine and uridine to wheat leaves infected with stem rust fungus (*Puccinia graminis* var. *tritici*).

Accumulation of photosynthetically assimilated ^{14}C in starch (Allen 1942; Inman 1962; Bushnell 1967), fructans and α-glucans (Holligan *et al.* 1973; Pung 1975), in tissues infected by obligate parasites has been reported. However, few workers have demonstrated the changes in lipid metabolism in plant tissues infected with obligately biotrophic fungi (Hoppe & Heitefuss 1974a,b,c, 1975a,b; Lösel & Lewis 1974; Lösel 1978). No microautoradiographic studies with reference to lipid in rust infection have been previously reported.

The movement of material from host tissue to mycelium of obligate parasites was studied by several workers. Reisener & Ziegler (1970) using ^{14}C, reported that amino acids and carbohydrates were transferred to rust spores from wheat leaf tissue. Mount & Ellingboe (1969) and Slesinski & Ellingboe (1971) found that movement of ^{32}P and ^{35}S from wheat to a powdery mildew fungus increased when the primary haustorium was forming. Mendgen & Heitefuss (1975) infected a bean leaf with tritium-labelled urediospores of *Uromyces phaseoli*, produced by feeding a bean plant with ^{3}H-orotic acid. In autoradiographs prepared 24 h after inoculation, no transfer of ^{3}H-orotic acid from the parasite to host cells could be detected. On the contrary, Ehrlich & Ehrlich (1970) reported the passage of

^{14}C-compounds from the pathogen (*Puccinia graminis* f.s. *tritici*) to host tissue of wheat in the infection center.

Mendgen (1979) supplied ^{3}H-lysine to bean leaves before inoculation with *Uromyces phaseoli* and demonstrated the uptake of label by mature haustoria but less by developing haustoria. When plants were inoculated with labelled urediospores, no transfer of tritium from fungal structures to host cells was detected. Favali & Marte (1973) fed tritiated glycine to bean plants, six days after inoculation with urediospores of *Uromyces phaseoli*, and reported dense labelling of the fungus. In general haustoria have been found to play an important role in the passage of material from host to parasite (Mount & Ellingboe 1969; Slesinski & Ellingboe 1971; Favali & Marte 1973; Mendgen 1979) or *vice versa* (Ehrlich & Ehrlich 1970). There was little evidence that the movement of substances between host and parasite may occur via the intercellular mycelium (Ehrlich & Ehrlich 1970; Favali & Marte 1973; Mendgen 1979). However, there is still very little information available concerning the path of interchange of metabolites between host and parasite and of the relative importance of intracellular hyphae and haustoria in this process.

1.6. Aims of the present study

Since the fungal haustorium is generally regarded as the principal nutritional interface between autotrophic host plants and most biotrophic fungi, there have been many structural investigations of haustoria in such associations, particularly in the powdery mildews

and the dikaryotic phase of rust fungi on their urediospore hosts (see reviews by Bushnell 1972; Bracker & Littlefield 1973). At the commencement of the present work no detailed comparison of the relationship of a single rust fungus with both its alternate hosts had been reported. No comparative physiological characterization of intracellular structures of monokaryon and dikaryon of a rust fungus has yet been published.

The purpose of the present investigation is to study:

1. The structure and morphology of *Puccinia poarum* on its alternate hosts;
2. The changes in tissues of *Tussilago farfara* during pycnial and aecial stages of rust infection;
3. The changes in tissues of *Poa pratensis* during uredial and telial stages of infection;
4. The physiology of the host-parasite interaction during monokaryotic and dikaryotic phases of infection using microautoradiography to study the movement of substrate from host to fungus, with particular reference to lipid metabolism.

CHAPTER 2

MATERIALS AND METHODS

2

MATERIALS AND METHODS

2.1 Maintenance of plant and parasite in the growth room

Plants of *Tussilago farfara* L. (coltsfoot) and *Poa pratensis* L. (smooth meadow grass) were maintained in a controlled growth room (day temperature 20°C, night temperature 15°C, light intensity 1100 lux from fluorescent tubes) and infected by *Puccinia poarum* Niels. as described by McGee *et al.* (1973). When pycnial pustules appeared, about 10 days later, nectar and pycniospores were transferred from one pustule to another, in order to induce dikaryotisation and the production of aecia. Infection of the aecial host, *Tussilago farfara*, was obtained by inducing the germination of the teliospores of this rust by alternate washing and drying. By direct application of the aeciospores or urediospores, the infection of the telial host, *Poa pratensis*, was obtained. To represent different stages and substages of the parasite's development, the following nomenclature was used:

P1: pycnial stage, aecia not visible, 7-14 days from inoculation, 3-5 mm diam.

P2: <50% of aecia dehisced, 14 days from inoculation 4-6 mm diam.

P3: >50% of aecia dehisced, more than 21 days from inoculation, 4-8 mm diam.

U1: uredial stage, 10 days from inoculation.

U2: closed uredium, 14 days from inoculation.

U3: opened uredium, more than 18 days from inoculation.

T1: fungal hyphae under lower epidermis, 18 days from inoculation.

T2: young teliospores; the spores appear as single-celled, aseptate, about 20 days from inoculation.

T3: mature telia, with brown pigment, more than 23 days from inoculation.

2.2 Preparation of material for light microscopy

2.2.1 Paraffin method

Small pieces (3-4 mm length) of infected and uninfected leaves of both hosts were fixed in acetic acid:alcohol (1:3 v:v) for 12 hours and then dehydrated in the following series of alcohol:water solutions:

10%	ethanol	for	2	hours
20%	"	"	"	"
30%	"	"	"	"
50%	"	"	"	"
70%	"	"	3	"
80%	"	"	"	"
95%	"	"	"	"

The pieces were then placed in absolute alcohol overnight, after which they were left in absolute alcohol:xylol with ratios of 3:1, 2:2 and 1:3 for $\frac{1}{2}$ hr in each. Specimens were then embedded in paraffin wax and sectioned on a Reichert microtome at 15 µm thickness. Sections were de-waxed in xylol and then taken through 100%, 95%, 80%, 70%, 50%, 30% and 10% alcohol (1-2 minutes each), then rinsed in water and stained with appropriate stain (see below).

2.2.2 Cryostat sections

A small piece (3-4 mm square) of fresh or fixed (as in 2.2.1) material was placed in a few drops of water or gum arabic as supporting and embedding medium and affixed by freezing it to the tissue holder inside the cryostat (Bright). The frozen tissue was sectioned at 10-15 µm thickness. Sections were picked up by touching a microscope slide against them. Usually, the temperature of the freezing chamber during sectioning was -30°C.

2.2.3 Freezing microtome sections

Sections (25-50 µm thick) of fresh plant material were cut by a freezing microtome using water or glue as embedding medium. The sections were removed from the knife with a camel's hair brush, placed in a small dish of water, then stained as below and examined.

2.2.4 Hand-cut sections (fresh material)

Hand sections of epidermis or upper mesophyll were cut in a plane parallel to the leaf surface, stained (see 2.2.6), mounted, usually in lactophenol, and examined.

2.2.5 Epon-embedded tissue (as prepared for transmission electron microscopy)

Semi-thin sections (0.5-1.0 µm thick) of infected and uninfected plant tissues were cut on Huxley (Cambridge) and Reichert ultramicrotomes from epon-embedded material, which had been prepared for

electron microscopy (see 2.3.1). Sections were picked up in a drop of water using a small piece of glass or a fine wire loop, transferred to a microscope slide, dried down using gentle heat, stained (see 2.2.6) and examined.

2.2.6 Staining for light microscopy (see Appendix for details of stains)

Generally, fixed (except in 2.2.5) and unfixed sections were stained with cotton blue in lactophenol (Shipton & Brown 1962). 1% aqueous safranin and 1% basic fuchsin were applied to stain the nucleus in fixed tissue, while Sudan IV in lactophenol or in lactophenol cotton blue was used to stain oil droplets. The sections from resin-embedded material were stained in 1% toluidine blue in 1% borax solution where a drop of stain was placed on the sections and warmed gently until the solution steamed, then washed off and dried with 50% alcohol solution. These sections were covered by a cover slip mounted in polymount and studied using a light microscope.

To detect callose, fresh sections from infected and uninfected tissues were used. Sections were stained and mounted in the staining solution of 0.005% water soluble aniline blue in 0.15 M phosphate buffer at pH 8.2 and examined after 30 minutes using a fluorescence microscope. In these conditions callose shows a yellow fluorescence (Currier 1957).

2.2.7 Section analysis

a) dimensions of the host cells and parasite structures were measured using a calibrated ocular micrometer.

b) Measurements were made of the number of haustoria and intracellular hyphae per host cell and the number of infected and uninfected cells of both hosts, each sample consisting of cells lying along a 300 µm transect line in a section parallel to the leaf surface.

c) Nuclei were measured in healthy cells from infected leaves and infected cells of comparable age in both hosts where their volumes were estimated using equations appropriate for spheres and cylinders.

d) The volumes of the epidermal cells, bundle sheath cells and fungal haustoria in infected *Poa* and areas of infected *Tussilago* upper mesophyll cells were calculated using the cylinder formula, while the formula for a sphere was used to find the volume of *Poa* mesophyll cells.

e) The numbers of chloroplasts in infected and uninfected mesophyll cells of both hosts were counted in 1 µm thick sections cut parallel to the epidermis from epon-embedded material. In sections (0.5-1.0 µm thick) from infected material prepared in this manner, the frequency of occurrence of septa in fungal intracellular structures was also studied.

2.3 Electron microscopy

2.3.1 Transmission electron microscopy (TEM)

Infected and uninfected tissues of both hosts were processed for electron microscopy (TEM) by a method based on Perera & Gay (1976).

The plant material was cut into pieces (1 mm square) under 2.5% (v:v) glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.0 (see Appendix) and left to fix at room temperature. After twenty-four hours, the specimen was washed in three changes of fresh buffer (20 minutes each), and then post-fixed in 1% (v:v) osmium tetroxide in 0.1 M sodium cacodylate buffer (Appendix) for one hour. After post-fixation, the tissue was washed in 2-3 changes of the same buffer (20 minutes each) and then dehydrated in the following series of alcohol:water solutions:

10%	ethanol	for	5	minutes	
20%	"	"	"	"	
30%	"	"	"	"	
45%	"	"	10	"	
60%	"	"	"	"	
75%	"	"	"	"	
95%	"	"	30	"	
100%	"	"	"	"	(2 changes)

The tissue pieces were then soaked in epoxy propane-absolute alcohol solution (50:50) and left for 30 minutes. Then they were transferred into pure epoxy propane (100%) and left for another 30 minutes. The pure epoxy propane was then replaced by a mixture of equal parts of epoxy propane and epon (1:1) and left for one hour in closed containers (embryo cups). The lids of the embryo cups were left slightly open, allowing epoxy propane to evaporate overnight. Then, after several changes in fresh resin over 2-3 days, tissue fragments were embedded in fresh resin (Appendix) in flat PVC embedding dishes or in embedding capsules, with the specimen number, written in Indian ink on a small slip of paper, placed in the centre

of each dish or capsule, and left in an oven at 60°C for forty-eight hours to polymerise.

The usefulness of the semi-thin sections (0.5-1.0 μm thick) and toluidine blue staining technique have been described by Mercer and Birbeck (1972). Such techniques were routinely used prior to electron microscopy. Under a binocular dissecting microscope, the desired area on the block was then trimmed into a flat-topped pyramid using a razor-blade. The sides of the pyramid were kept parallel. Sections were examined with the light microscope and photographed using a Zeiss Ultraphot microscope.

Ultra-thin sections were cut by the same ultramicrotomes mentioned above. Silver or gold sections were picked up on a dull surface of formvar (polyvinyl formaldehyde)-coated copper grids, which were prepared by a method outlined by **Nunn** (1970) and Juniper *et al.* (1970), using 0.6% (w:v) formvar in chloroform. A piece of filter paper was slipped between the tips of a pair of forceps to remove the film water on the grid.

Ultra-thin sections were double-stained on a wax plate placed in a petri dish. Pellets of sodium hydroxide were placed in the petri dish to remove carbon dioxide from the environment. A drop of 2% aqueous uranyl acetate (Juniper *et al.* 1970) was pipetted on the wax plate and the mounted grids were gently floated, with the sections facing down on the drop of the stain. The dish was immediately covered with a lid. The sections were left for 30 minutes in uranyl acetate and then washed in a gentle stream of glass distilled water and dried on filter paper. The same method was adopted for lead citrate staining, the sections being placed in

Reynolds' lead citrate solution (Reynolds 1963, see Appendix) and left for 10-20 minutes, then washed and dried as for uranyl acetate staining. The sections were examined and photographed using AEI 6B Corinth and Phillips electron microscopes.

2.3.2 Scanning electron microscopy (SEM)

Leaf segments (3-5 mm) of both hosts were prepared for scanning electron microscopy by a method based on Mercer & Birbeck (1972). Segments with cut edges were fixed for two hours in 2.5% v:v glutaraldehyde in 0.1 M sodium cacodylate (see Appendix) buffer at pH 7.0, dehydrated directly through an ethanol series (25, 50, 75, 100% for 10 minutes each) and then passed through a similarly-graded series of acetone solutions (for 10 minutes each). Specimens were dried using a Polaron E3000 critical point drying apparatus, then flushed with liquid CO_2 and left soaking in liquid CO_2, after all acetone had been flushed off, for 1 hour. The dried specimen was mounted on a special specimen holder (SEM stubs) with Dag 915 (electrical silver paint) and then coated with gold in an Edwards S-150 sputter coater, in an atmosphere of Argon 2 x 2 minutes, at 45 milli-amps. The specimen was then examined and photographed using Phillips SEM-501.

Using this method, the morphology of spores and sori, the growth of fungal mycelium in the host and the presence of certain fungal features such as uredial and telial paraphyses were studied.

2.4 Autoradiographic studies

2.4.1 Labelling and processing the tissue

^{3}H-glycerol was obtained from the radiochemical centre, Amersham, with specific activity of 2.5 Ci/m Mole. Small portions, 2 cm x 1.5 cm from infected *Tussilago* leaves (with vein passing through infected and uninfected parts) at P2 stage of parasite development (14 days from inoculation), and from infected *Poa* leaves showing uredial stage (about 18 days from inoculation) were selected. These portions were fed separately with various doses of ^{3}H-glycerol, i.e. 50, 100 and 200 µCi/500 µl distilled water. It was found that the 200 µCi/500 µl distilled water gave the most satisfactory results in both plants. The bottom 4-5 mm of the uninfected part of the leaf portion was immersed in the feeding solution placed in a small vial. Care was taken to avoid any contact between the solution and the areas to be sampled. The leaf portion was allowed to take up all the ^{3}H-glycerol solution (approximately 7 hours), then distilled water was added to the vial and the leaf left in the dark for a chase period overnight. To ensure uniform transpiration and photosynthesis during the feeding time, leaves were exposed to natural daylight and a slow stream of air was allowed to circulate around the surface of the leaf. Aeration continued in darkness. The leaf portion was then placed in 2.5% (v:v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0 and the infected and uninfected parts were cut under the fixative into small segments, 1 mm square. These segments were fixed in glutaraldehyde separately for 24 hours and post-fixed with 1% (v:v) osmium tetroxide

in 0.1 M sodium cacodylate buffer. The fixed segments were then processed for light and electron microscope autoradiography as described in 2.3.1. The whole leaf portions which had been fed and fixed as above, were prepared for macroautoradiography.

2.4.2 Macro-autoradiography

After fixation, leaf portions were pressed flat and dried at 80°C. They were then fastened to hardboard sheet covered with chromatography paper using sellotape. They were then covered with Melinex film to prevent chemography. In the dark room, supplied with safe light, specimens were covered with X-ray film and taped securely. A second sheet of hardboard covered with chromatography paper was placed over X-ray film. The whole preparation was wrapped with black polythene and left to expose for 10 days. At the end of the exposure time, the X-ray film was then developed for 5 minutes in PQ developer, washed and fixed in the usual way.

2.4.3 Autoradiographic preparation for light microscopy

One micron thick, resin sections of infected and uninfected tissues were cut with glass-knives on a Huxley (Cambridge) ultramicrotome. Solvent vapour (acetone or chloroform) was used to expand the sections on the water bath. Sections were then transferred to clean glass microscope slides (see Appendix) previously coated with chromated gelatin (1 g of gelatin and 0.1 g of chrome alum dissolved in distilled water and the solution made up to 100 ml). They were dipped into a liquid photographic emulsion (Ilford G5) (Williams 1977), dried and left to expose for 14 days in sealed light-tight boxes in a refrigerator at 4°C.

Liquid emulsion was prepared by mixing 30 ml of molten G5 emulsion (previously stirred very slowly, e.g. one revolution per second) with 30 ml of distilled water which contained a drop of glycerol (to increase the elasticity of the emulsion) in a clean dipping jar (e.g. a truncated 250 ml measuring cylinder). The contents of the dipping jar were gently stirred in a thermostatically controlled water bath at 43°C and left for 5 minutes to settle. In order to get a uniform coating of the emulsion, the slides must be withdrawn steadily from the dipping jar, kept for a few seconds vertically, standing up against a support to drain, then laid flat on a cooled surface to set. The preparations were subsequently developed in diluted Ilford phenex developer (60:40 developer: distilled water) for 8 minutes, washed in distilled water for 8 minutes, fixed in sodium thiosulphate (hypo) for 8 minutes, and again washed in distilled water for 8 minutes. All operations up to this point were done in the dark-room under a recommended safe light. Finally, the slides were stained with 0.5% solution of toluidine blue in 0.5% borax, differentiated with 50% ethanol, mounted in polymount and viewed by bright-field and dark-field microscopes.

2.4.4 Examination and assessment of autoradiographs

The distribution of silver grains over tissue, in autoradiographs prepared as above, was examined with the light microscope. Photographic records were made using a Vickers M17 metallurgical microscope, with dual illumination, allowing an appropriate balance of transmitted light and vertical incidence illumination, which is reflected by silver grains in the emulsion over the specimen.

Quantitative assessment of autoradiography by measurements of reflectance was carried out using the system described by Goldstein & Williams (1971). This consists of a Leitz 'Ortholux' microscope with a vertical incidence illuminator, a Leitz MPV microscope photometer and a 60x oil immersion objective, linked with a photomultiplier tube, stabilized high tension power supply and a multirange meter, supplied by Evans Electroselenium Ltd. Since reflectance from the specimen and optical system are reduced by the use of polarized light, a rotatable polarizer is situated in the illuminating beam and a non-rotable analyser in the microscope body, just above the beam splitter of the incident illuminator (Goldstein & Williams 1974).

Reflectance measurements on circular areas of about 50 μm diameter were made on five sections from each of five autoradiograph slides, prepared from each of the various regions of healthy and infected tissue of both hosts of *Puccinia poarum*. Mean values of five replicate areas per section were recorded for the individual features to be compared and corrected for background by subtracting the mean of five similar areas adjacent to the tissue. A completely fogged specimen of Kodak AR10 stripping film was used as reflectance standard. Emulsion-coated sections of unlabelled leaves did not give reflectance values above the background level.

A diagram indicating the positions of tissue sampled, infected regions and points of uptake of ^{3}H-glycerol (Fig. 8.1) is included with plates of the resulting autoradiographs in Chapter 8.

2.5 Abbreviations used in Plates

Host

BS, bundle sheath cell; Ca, callose; CC, companion cell; Ch, chloroplast; Cr, crystal; E, epidermal cell; Ech, euchromatin; EN, endodermal cell; ER, host endoplasmic reticulum; Fr, fibre; g, Golgi bodies; G, grana; Hch, heterochromatin; HN, host hucleus; HPL, host plasma membrane; NV, host vacuole; HW, host cell wall; IG, intergranal lamellae; L, lipid drop;, M, host mitochondria; MB, host microbody; MC, mesophyll cell; Nu, host nucleolus; OS, osmiophilic globule; Pd, plasmodesmata; Ph, phloem; PP, phloem parenchyma; R.E.R., rough endoplasmic reticulum; Se, sieve tube; St, starch grain; VS, vascular strand; Wi, wall ingrowths; X, xylem treacheary element; XY, xylem; XP, xylem parenchyma.

Fungus

a, aeciospore; A, adhesive material; AI, aeciospore initial; Ap, appiculus; As, aeciosporophore; B, basidiospore; C, collar; D, depression; er, fungal endoplasmic reticulum; F, flexuous hypha; Fpl, fungal plasma membrane; Fs, fungal stroma; FW, fungal wall; gr, germ pore; H, haustorium; HB, Haustorial body; HMC, haustorium-mother cell; Hy, hymenium; I, intercellular hypha; Ia, intracellular hypha; IMC, intracellular hypha-mother cell; IP, invaginated host plasma membrane (comparable to extrahaustorial membrane); Lo, lomasome; m, mitochondria; Mb, fungal microbody; mx, matrix region (equivalent to extrahaustorial matrix, Littlefield & Heath 1979); n, fungal nucleus; N, haustorial neck; NB, neckband; nu, fungal nucleolus; P, pore apparatus; Pc, pycniospore; Pr, peridium; Ps, pycniosporophore; Py, pycnium; R, refractive granule; S, septum; Sm, sticky matrix; T, localized thickening; TP, telial paraphyses; Ts, teliospore; U, urediospore; UP, uredial paraphyses; US, urediosorus; V, vacuole; Vm, vesicle with membranes; Ve, vesicle (empty or with dense material).

CHAPTER 3

MORPHOLOGY AND ONTOGENY OF SPORES AND SORI

3

MORPHOLOGY AND ONTOGENY OF SPORES AND SORI

Measurements of spores and sporulating structures of *Puccinia poarum* are recorded in Table 3.1.

3.1 Pycnia and pycniospores

About one week after infection of *Tussilago* leaves by basidiospores, released after germination of teliospores, a mass of prosenchymatous tissue (or proto-pycnium) (Fig.3.1) is observed in the upper mesophyll tissue. This protopycnium is distinguished from the proto-aecium by occupying smaller areas in the mesophyll tissue. In the development of the mature pycnium cells, closely packed pycniosporophores, elongated, uninucleate cells, 29 µm in length, arise from a peripheral layer of pseudoparenchymatous cells (Figs. 3.2, 3.3, 3.10) and produce pycniospores which accumulate in a dense mass at the centre of the flask-shaped pycnium. The formation of the pycniospore is marked by a swelling of the pycniosporophore apex (Figs. 3.2, 3.4). Pycniospores are smooth, pear-shaped (Figs.3.5, 3.7) and average 4.8 µm in length. At maturity, thousands of these spores are exuded (Figs. 3.6, 3.7, 3.8) through the pycnial ostiole in a sticky matrix (Fig. 3.9). The mature pycnium (Fig. 3.6, 3.10) is adaxial, flask-shaped, 137 µm length, 124 µm width and surrounded by pseudoparenchymatous tissue (Figs. 3.2, 3.3, 3.10). The mouth

of the pycnium, which penetrates the upper epidermis is lined by unbranched pointed hairs, the periphyses (Figs. 3.7, 3.8). Among the periphyses are branched hyphae, the flexuous hyphae (Figs. 3.8, 3.11, 3.12) which, in older pycnia, are mostly longer than the periphyses (Figs. 3.11, 3.12).

Both light microscopy (Fig. 3.13) and scanning electron microscopy (Fig. 3.14) reveal the transformation of periphyses into flexuous hyphae in mature pycnia of this rust. Such transformation is clearly observed as hyphal growth at the apices of periphyses, where differences in morphology are seen between the converted periphyses and the newly-formed flexuous hyphae on their tips.

Lipid, in the form of yellow refractive droplets when stained by cotton blue in lactophenol and staining red with Sudan IV in lactophenol, was more abundant in cells of the pycnium than any other fungal structures in its vicinity. Measurements of the external features of the pycnial stage are recorded in Table 3.1.

3.2 Aecia and aeciospores

Aecia of *Puccinia poarum* originate from a pseudoparenchymatous tissue (proto-aecium) which develops in the lower mesophyll of *Tussilago* leaves (Figs. 3.15, 3.16). Nuclear staining indicates that the proto-aecium consists of monokaryotic cells (Fig. 3.15). From below this tissue, a hymenium of aeciosporophores arises as elongated cells (Figs. 3.17, 3.18), which give rise to aeciospore initials. The cells of this hymenium are of much greater size (27.3 μm in length, 7.1 μm in diameter) than those of the dense

prosenchymatous stroma, which develops below the hymenial layer, and similar in diameter to newly formed aeciospores. At various points of the junction of this prosenchymatous stroma and the hymenium, hyphal fusions appear to be present (Figs. 3.17, 3.18). The sporophore and aeciospore initial are separated by a septum (Fig.3.17). These cells as well as the fungal tissue below them are highly vacuolated (Figs. 3.17, 3.19). The thickening shown in the wall of the aeciosporophores (Fig. 3.17) corresponds to the thickened collar described in aeciosporophores of *Puccinia sorghi* by Rijkenberg & Truter (1974b). Aeciospore initials divided to form immature aeciospores and intercalary cells (or disjunctor cells, as used by Kozar & Netolitzky 1975) (Figs. 3.17, 3.20). Aecia are mostly abaxial, cup-shaped (aecidioid as defined by Littlefield & Heath 1979) and average 266 µm and 404 µm in width when closed and opened respectively (Figs. 3.22 to 3.26). Aeciospores are oblong or ellipsoid and average 20.3 µm x 23.6 µm (Figs. 3.27 to 3.31). Several germ pores occur on each aeciospore (Figs. 3.21, 3.29, 3.30, 3.31). The wall of the aeciospore is colourless (Figs. 3.21, 3.33) and verrucose-echinulate (Figs. 3.27 to 3.31). Examination by scanning electron-microcrope shows that coglike ornamentations (Figs. 3.27, 3.28) (Littlefield & Heath 1979) and refractive granules (Figs. 3.28, 3.29, 3,31, 3.33) are present on the surface of the aeciospores. The refractive granules appear to lie over germ pores. In Fig. 3.31, some germ pores appear to be filled to varying extents by material which could represent stages in the development of refractive granules, while other pores appear to have lost their plug material. From their appearance (Figs. 3.29, 3.30) it is possible that the refractive

granule's function may be to enable the spore and germ tube to stick to the leaf surface of the host. Those seen adhering to germ tubes on the host leaf surface at some distance from the spore may have been carried there on the growing germ tube.

The aecium and aeciospores are enclosed by the peridium (Figs. 3.22, 3.23, 3.32, 3.34), in which each peridial cell measures 26.4 µm x 23.9 µm (Table 3.1). Some ornamentation of the surface of the peridial cell is seen (Figs. 3.32, 3.34). Oil droplets are abundant in both aeciospores and peridial cells. A dense layer (69.3 µm thick) of fungal cells is seen around each aecium (Fig.3.35), closely in contact with the peridium at its base but separated from it by a space in the upper region of the aecium. Scanning EM reveals the characteristically rounded outer wall surface of the aecium at the time of its dehiscence. Stages in emergence and dehiscence of aecia are shown in Figs. 3.24, 3.25, 3.26, 3.32. In Fig. 3.32, the peridium has opened, exposing the aeciospores (cf. Fig. 3.23), and the margin appears as five teeth, bending outwards. Aecia and pycnia also occur on the petioles of *Tussilago* leaves. Measurements of external features of the aecial stage are reported in Table 3.1.

3.3 Uredia and urediospores

On *Poa* leaves, uredia are mostly adaxial (Fig. 3.36). The earliest stage of development shows a mass of dikaryotic pseudoparenchymatous tissue (termed 'uredial initial' by Littlefield & Heath 1979) which is formed under the host epidermis (Fig. 3.37).

Sporogenous cells, stalked urediospores and paraphyses arise from this sporogenous tissue (Fig. 3.38). Urediospores (mean dimensions 24.3 µm x 19.5 µm) are mostly obovoid, ellipsoid, with colourless, echinulate walls (e.g. Figs. 3.36, 3.39-3.44), showing an average of 5 germ pores, which are much more easily seen with the light microscope than with the scanning electron microscope. This discrepancy could indicate that the germ pores are covered by the outermost layer of the urediospore wall and are only detected with transmitted light. A section of a urediosorus from resin-embedded material (Fig. 3.46) appears to support this suggestion. Such covered pores could account for the small depression seen in some scanning EM pictures of urediospores (Figs. 3.40, 3.41). The spore stalks have a mean length of 25 µm. Large oil bodies are present in urediospores, together with a number of smaller lipid drops (Figs. 3.43, 3.45-3.47). In fixed and resin-embedded sections, masses of small lipid drops are seen in the peripheral cytoplasm of urediospores (Figs. 3.46, 3.50). On germination, urediospores produce two or more germ tubes (Figs. 3.43, 3.44) with dense cytoplasm and numerous oil drops in their distal regions. During this process the spores and proximal regions of the germ tubes become highly vacuolated.

Until maturity, the uredia remain covered by the host epidermis (Fig. 3.47) which finally splits open (Figs. 3.40, 3.41) allowing the release of the urediospores. The development of urediospores is accompanied by the growth of elongated, colourless, capitate, non-septate paraphyses (Figs. 3.48-3.55) which may play some part in the rupturing of the epidermis or the release of the spores. All

mature uredia examined were found to have paraphyses, these structures becoming larger, more prominent and more numerous as the uredium expands. The heads of fully grown paraphyses are approximately spherical and similar in size to the urediospores (Figs. 3.52-3.55) from which they also differ in their smooth, unornamented surface and less dense cytoplasm (Figs. 3.48, 3.51). Ornamentations are seen on the surface of urediospores (Figs. 3.40-3.56) in the form of spines, each situated in a circular depression encircled by an annulus (Figs. 3.55, 3.56). Urediospores appear to be readily dislodged from their stalks. In favourable views, scanning EM pictures show a scar on the urediospore corresponding exactly in appearance and size with the exposed ends of urediospore stalks which have lost their spores (Figs. 3.41, 3.52). Such scars show a thickened rim and a central pore region. Teliospores occasionally arise among urediospores (Figs. 3.57, 3.58), indicating the connection between uredial and telial mycelia. Light microscope examination reveals that a layer of fungal cells (peridium) covers the urediospores (Figs. 3.59, 3.60, 3.61, 3.62), which is frequently damaged as the spores rupture the host epidermis. The outline of the walls of peridial cells is readily observed in surface view of epidermal fragments from infected *Poa* leaves (Fig. 3.61), but not easily detected in transverse sections of the urediosori (Figs. 3.36, 3.47, 3.59, 3.60). It is suggested that their inner walls break down during the maturation of the urediospores leaving their thickened, outer tangential walls adhering to the undersurface of the host epidermis.

3.4 Telia and teliospores

Telia are mostly abaxial, elongated, covered by the host epidermis and (Figs. 3.63, 3.54) variable in size. In transverse section they show tightly packed groups of two-celled teliospores on very short stalks, separated from adjacent groups by non-septate paraphyses (Figs. 3.63, 3.64, 3.68, 3.69) of the same dark brown colour as the teliospores. As a result of this very tight packing during their growth mature teliospores are somewhat variable in shape (Fig. 3.66), often slightly curved and angular in cross section.

As in urediospore formation, teliospores with their persistent pedicels and paraphyses arise from a dense, dikaryotic sporogenous tissue (Figs. 3.63, 3.65) lying between the epidermis and mesophyll tissue of *Poa* leaves. The spore is separated from its pedicel by a septum (Figs. 3.65, 3.66). A striking accumulation of lipid is found in the sporogenous tissue, pedicels, and teliospores (Figs. 3.65, 3.67). Teliospores (averaged 54.3 µm x 17.2 µm) are mostly elongated, obovoid or oblong-clavate and either truncate or rounded at the apex (Figs. 3.66, 3.68). The young teliospores are initially single-celled but, at maturity, the spore is divided into two cells by a septum (Figs. 3.66, 3.67). Fig. 3.67 shows movement of material between these two cells through a septal pore of an immature teliospore. The surface of the teliospores generally appears smooth, but at higher magnification, scanning electron microscopy reveals a shallow rugulose ornamentation (Fig. 3.68).

Paraphyses enclosing groups of spores in telia are easily observed with both light microscope (Figs. 3.63, 3.69) and scanning electron microscope (Figs. 3.64, 3.68). They are brownish in colour, elongated and capitate (Figs. 3.68, 3.69). After germination of teliospores, basidiospores are released and infect *Tussilago* leaves. Basidiospores are ovate to pyriform, smooth-surfaced and attached to a conical apiculus (Fig. 3.70). The measurements of the external features of uredial and telial stages are recorded in Table 3.1.

Table 3.1 Measurements* of the external features of pycnial, aecial, uredial and telial stages of *Puccinia poarum*.

Features	Dimensions (µm)	Number of samples
Pycnium (LxW)	137.0 (±3.5) x 124.0 (±3.4)	25
Pycniosporophore (L)	28.8 ± 0.7	50
Pycniospore (L)	4.8 ± 0.3	25
Flexuous hypha (L)	149.0 ± 9.3	25
Periphyses (L)	95.0 ± 2.2	25
Closed aecium (W)	266.0 ± 13.4	10
Opened aecium (W)	404.0 ± 8.6	15
Aeciosporophore (LxW)	27.3 (±0.5) x 7.1 (±0.5)	50
Aeciospore (LxW)	20.3 (±0.2) x 23.6 (±0.4)	25
Peridial cell (LxW)	26.4 (±0.7) x 23.9 (±1.0)	10
Fungal layer around aecium (T)	69.3 ± 3.3	15
Urediospore (LxW)	24.3 (±0.9) x 19.5 (±0.3)	25
Urediospore stalk (L)	25.2 ± 1.3	20
Germ pores in urediospore (n)	4.5 ± 0.4	15
Teliospore (LxW)	54.3 (±1.0) x 17.2 (±0.7)	15
Pedicel (L)	15.3 ± 0.6	15

*Measurements made using light microscope on sections of fresh material, mounted in lactophenol.

L, length; W, width; T, thickness; n, number; ±, Standard error of mean

CHAPTER 4

QUANTITATIVE ASSESSMENT OF GROWTH OF *PUCCINIA POARUM* IN TISSUES OF THE ALTERNATE HOSTS

4

QUANTITATIVE ASSESSMENT OF GROWTH OF *PUCCINIA POARUM* IN TISSUES OF THE ALTERNATE HOSTS

4.1 Growth of *P. poarum* on *Tussilago farfara*

The earliest stage of the haploid phase of *P. poarum* on the pycnial-aecial host, *Tussilago farfara* shows an active mycelial growth radiating out from a dense fungal mass or proto-pycnium, formed in an intercellular space within the upper mesophyll (Fig. 3.1), and rapidly colonizing the mesophyll tissue of *Tussilago* leaves forming proto-aecia in the lower mesophyll (Figs. 3.15, 3.16). The development of these structures into pycnia and aecia has been described in Chapter 3. Nuclear staining reveals that the bulk of the fungal mycelium external to the aecium in pycnial-aecial stages is monokaryotic and that dikaryotic cells are only observed in the prosenchymatous fungal tissue at the base of the aecium.

In sections through the margin of the developing pycnial-aecial infections, cut parallel to and just below the epidermis, intercellular hyphae are seen to radiate freely through the mesophyll (Fig. 4.1). These hyphae are septate, of average diameter 3 μm, and vary in the length of their cells. The tip of a hypha becomes closely appressed to the mesophyll cell forming a terminal cell, the intracellular hypha-mother cell. At the colony margin, the mother cells give rise to intracellular hyphae, which are slightly constricted at the point where they penetrate the host cell wall. These intracellular hyphae

are designated type X (Fig. 4.2). In the older parts of the pycnial-aecial pustule (pustule centre), similar sections show intercellular hyphae growing along the host cell wall in the vicinity of a maturing pycnium, occasionally penetrating host cells with an apparently unmodified, less specialized intracellular hypha (Type Y), which is not constricted at the point of penetration (Fig. 4.3). Both X and Y types of intracellular hyphae are unbranched, irregular in outline and occasionally septate, but are not cut off by a septum at the point of entry to the host cell. Semi-thin sections (0.5-1.0 μm thick) from epon-embedded material reveal that more septa occur in intracellular hyphae during the later stages (Fig. 4.4) of pycnial-aecial infections than in younger stages. Lipid accumulates in both types of intracellular hyphae (Figs. 4.3, 4.5). In addition to those in mesophyll cells, intracellular hyphae are observed in epidermal cells of *Tussilago* (Fig. 4.6). Features of size and morphology of intracellular hyphae at different stages of pycnial-aecial development are compared with those of haustoria of uredial and telial stages in Table 4.1. The length of intracellular hyphae in young pycnial-aecial infections is approximately twice that of haustoria formed by the young uredial and telial stages. The growth of inter- and intracellular hyphae in pycnial-aecial pustules developing in the petioles of *Tussilago* leaves is similar to that in the lamina of the leaf.

The intensity of infection of host cells by intracellular hyphae at different stages of development of the monokaryon on *Tussilago* is compared with that by haustoria of the dikaryon on *Poa* in Table 4.2. This study indicates that approximately 60% of the upper mesophyll

cells in infected regions of *Tussilago* leaves are penetrated by one or, occasionally, two intracellular hyphae (Fig. 4.7a), but rarely by more than two (Fig. 4.7b).

4.2 Growth of *P. poarum* on *Poa pratensis*

About one week after infection of *Poa* leaves, the early stages of uredia form just below the upper epidermis. The vegetative growth of the parasite in the intercellular spaces of *Poa* leaves is less extensive than that of the pycnial-aecial stages in *Tussilago*, although the hyphae form an increasingly dense intercellular network (Fig. 4.8) close to urediosori and teliosori as infection advances. The diameters of the intercellular hyphae and haustoria are similar.

The morphological features of haustoria formed by uredial infections are recorded in Table 4.1. The haustoria are more limited in size than the monokaryotic intracellular hyphae, smooth in outline, with a clearly defined matrix (Figs. 4.9, 4.14) (corresponding to the term "extrahaustorial matrix" used by Littlefield & Heath 1979). The wall of the haustorium-mother cell stains more intensively with cotton blue in lactophenol than the rest of the hypha which is terminates and can be recognized before penetration as it comes into contact with the host cell wall (Fig. 4.10). Like uredial haustoria of other rusts (Rice 1927; Coffey *et al.* 1972) those of *P. poarum* have a characteristically constricted neck region which extends some distance within the lumen of the host cell (Figs. 4.10, 4.12). In infected regions of *Poa* leaves, numerous haustoria are observed in mesophyll cells (Figs. 4.9, 4.13, 4.14) and in epidermal cells

(Figs. 4.11, 4.12), including the bulliform cells of the adaxial epidermis above the midrib region. Multiple infections of epidermal cells are common (Fig. 4.11).

In the vicinity of uredia, more than 70% of cells seen in longitudinal sections of the mesophyll of *Poa* leaves, cut parallel to the leaf surface, are penetrated by haustoria (Table 4.2) and about 25% of these by two or three haustoria per cell (Figs. 4.9, 4.13, 4.14). When telia are present, 75% of the mesophyll cells are invaded by haustoria, more than one-third of the infected cells containing two or more haustoria. Although the uredial and telial stages form less extensive growth of the intercellular mycelium than that of the pycnial-aecial infections, Table 4.2 shows that the percentage of cells penetrated by haustoria is more than that of *Tussilago* cells penetrated by intracellular hyphae of the monokaryon. Since the host cells of *Poa* are much smaller than those of *Tussilago*, this implies a considerably higher number of host cell penetrations per unit area of leaf by the dikaryon than by the monokaryon.

At least three types of haustoria are found in the uredial and telial phases of development: a, short and rod-like; b, L-shaped; c, C-shaped with the tip lying near the neck (Fig.4.15a,b,c). The rod-like haustoria (Type a) are the most frequent type at all stages of development of uredial infections, while b- and c-types are occasionally observed in early uredial substages but increase in frequency later in infection. Measurements of lengths of each haustorial type at successive stages of uredial and telial development are compared in Table 4.3 The L-shaped and C-shaped forms of haustoria increase consistently in length throughout the growth of uredia.

Generally similar results are obtained from measurements of haustoria during the development of telia but with some reduction in the number of significant differences as infection progresses. Table 4.3 shows a close similarity in size of haustoria of corresponding age and type between uredial and telial infections. Haustoria also increase in width with age of infection (Table 4.4).

The volumes of haustoria in different types of *Poa* leaf cells, which are penetrated by one or more haustoria, are recorded in Table 4.5 and related to the volumes of the host cells. A negative correlation appears to exist between haustorium size and host cell size. In bundle sheath cells, haustoria occupy a mean 1.5%, 2.6% and 3.3% respectively of the mean cell volume of just under 16000 μm^3 when one, two or three haustoria are present per cell. In upper mesophyll cells, with a mean cell volume of 18000 μm^3, haustoria occupy 1.2% of the cell volume, in single infections and 1.5% where two or three haustoria have penetrated. The much larger epidermal cells of *Poa* have, however, only 0.07% of their volume occupied by haustoria, in the case of infection by one or two haustoria per cell, and 0.12% where three haustoria are present.

With the onset of teliospore development, the cell walls of the intercellular hyphae show a brown pigmentation, similar to but less dense than that of the teliospores and telial paraphyses. Although the appearance of the fungus in the vicinity of uredia and telia shows some differences in amount and colour of cells, these stages are frequently present together in a single section of a leaf of *Poa* and thus appear to develop in parallel rather than in succession.

Table 4.1 Structural features of intracellular organs of *Puccinia poarum* in mesophyll cells of its alternate hosts.

	Pycnial-aecial stages on *Tussilago farfara*			Uredial-telial stages on *Poa pratensis*					
	Margin		Centre						
Age of pustule	Young (P_1)	Mature (P_3)	Mature (P_3)	Young (U_1)			Mature (T_3)		
Type of intracellular organ	x	x	y	a	b	c	a	b	c
Mean length (μm)*	20.9	25.0	32.5	9.4	13.0	14.3	12.8	18.1	21.5
Number of organs counted	25	25	25	26	20	12	26	26	26
Description	Slight constriction at point of entry		No constriction at point of entry	Constricted, well-defined neck region					
	Irregular outline and growth habit			Smooth outline with straight or curved body					
	Occasionally associated with host cell nucleus			Frequently in close proximity to host nucleus					
	Mother cell not distinguished from rest of intercellular mycelium			The wall of the mother cell stained more intensively than intercellular hypha					

* = Standard errors of means in all cases below 1% (each figure based on measurements of cells from 10 pustules).

Table 4.2 Quantitative comparison of intensity of infection at successive stages of development of *Puccinia poarum* on leaves of *Tussilago farfara* and *Poa pratensis*.

	Mean number of cells containing each number of intracellular structures				Mean number of cells per sample	Percentage of cells infected
Number of intracellular fungal structures in each host cell	1	2	3	4		
T. farfara						
Young pycnial stage (P_1)	2.55 ± 0.19	0.25 ± 0.10	0	0	4.70 ± 0.18	59.58
Mature pycnial stage (P_3)	3.05 ± 0.17	0.40 ± 0.15	0	0.05 ± 0.05	5.65 ± 0.15	61.95
P. pratensis						
Uredial stage (U_3)	5.10 ± 0.38	1.40 ± 0.18	0.30 ± 0.15	0	9.40 ± 0.38	72.34
Telial stage (T_3)	4.60 ± 0.32	2.10 ± 0.39	0.40 ± 0.13	0	9.40 ± 0.22	75.53

Values are means of 20 counts ± standard errors of means, each sample consisting of cells lying along a 300 μm transect line in a section through the upper mesophyll, parallel to epidermis.

Table 4.3 Lengths (μm) of haustoria of *Puccinia poarum* at successive stages of development of uredia and telia[†]

Mean type	length	U_1 a	U_1 b	U_1 c	U_2 a	U_2 b	U_2 c	U_3 a	U_3 b	U_3 c
a	9·4									
b	13·0	**								
c	14·3	**	—							
a	11·3	**	—	—						
b	17·8	***	***	*.	***					
c	22·6	* *	***	***	***	* **				
a	12·7	***	—	—	—	***	***			
b	19·9	***	***	***	***	* *	*	***		
c	25·3	* *	***	* *	***	** *	*	***	** *	

Mean type	length	T_1 a	T_1 b	T_1 c	T_2 a	T_2 b	T_2 c	T_3 a	T_3 b	T_3 c
a	10·0									
b	15·4	***								
c	15·3	* *	—							
a	11·0	—	***	* *						
b	17·2	***	—	—	***					
c	18·2	***	***	*	***	—				
a	12·8	* *	***	—	* *	***	* *			
b	18·1	***	* *	—	***	—	—	***		
c	21·5	***	***	***	***	***	***	***	**	

[†] Based on measurements of more than 20 haustoria except where otherwise indicated. cU_1 and cT_1 based on measurements of 10-15 haustoria.

*, **, *** indicate differences in length estimated to exceed 0.05, 0.01 and 0.001 levels of significance.

— indicates below 0.05 level of significance.

Table 4.4. Mean width (μm) of haustoria at successive stages of development of uredia and telia.†

	Haustorium type	U_1	U_2	U_3
Uredium	a	3.2 ± 0.1	3.5 ± 0.1	3.7 ± 0.2
	b	3.5 ± 0.1	3.9 ± 0.1	4.2 ± 0.2
	c	*3.3 ± 0.1	3.7 ± 0.2	4.1 ± 0.1
		T_1	T_2	T_3
Telium	a	*3.4 ± 0.3	3.4 ± 0.1	3.6 ± 0.2
	b	*3.4 ± 0.1	3.5 ± 0.2	4.3 ± 0.1
	c	*3.4 ± 0.2	3.5 ± 0.2	3.9 ± 0.2

† Mean values (± standard error) based on measurements of more than 20 haustoria, except where otherwise indicated.

* Based on measurements of 10-15 haustoria.

Table 4.5 Relationship of number and size of haustoria of *Puccinia poarum* at uredial stage to the volume of infected host cells of *Poa pratensis*.

		Volume of haustoria (μm^3)					
		One haustorium per host cell[1]	Two haustoria per host cell[2]		Three haustoria per host cell[3]			Volume of host cell (μm^3)[1]
Epidermis		127.5±12.4	92.0±12.6	46.8±3.5	122.1±33.3	50.1±4.9	48.5±5.3	192692.5±17518.7
Total volume of haustoria / Host cell volume	%	0.07	0.05	0.02	0.06	0.03	0.03	
Mesophyll		219.7±16.9	170.0±16.7	108.1±9.6	112.4±7.8	91.2±16.9	65.0±17.0	18428.7± 1879.4
Total volume of haustoria / Host cell volume	%	1.2	0.9	0.6	0.6	0.5	0.4	
Bundle sheath		241.2±17.3	252.8±20.3	153.3±9.0	276.3±73.2	151.5±22.0	97.5±13.1	15853.9± 1535.3
Total volume of haustoria / Host cell volume	%	1.5	1.6	1.0	1.7	1.0	0.6	

Numbers of host cells on which mean values (± standard errors) are based: 1 = 30 cells;
2 = 20 cells;
3 = 10, 5 and 5 cells in epidermis, mesophyll and bundle sheath, respectively

In all cases mature uredial-telial pustules were used (24 days after inoculation)

CHAPTER 5

ELECTRON MICROSCOPIC STUDIES OF INTER- AND INTRA-CELLULAR STRUCTURES OF *PUCCINIA POARUM*

5

ELECTRON MICROSCOPIC STUDIES OF INTER- AND INTRA-CELLULAR STRUCTURES OF *PUCCINIA POARUM*

5.1 Intercellular hyphae

The pycnial and aecial stages of *P. poarum* show an extensive growth of monokaryotic fungal hyphae in the intercellular spaces of *Tussilago* mesophyll tissue (Fig. 5.1). Hyphae of the haploid mycelium are often closely associated with the host cell wall; they frequently penetrate the middle lamella region separating contiguous cells and may grow within the host cell wall or parietally(Fig. 5.2), becoming embedded in a thick layer of host wall material which protrudes deeply into the lumen of *Tussilago* cells. In the vascular bundles of *Tussilago* leaves, where intercellular spaces are absent, hyphae frequently grow within the host cell wall (see Fig. 5.7). A more limited growth of dikaryotic mycelium develops in the intercellular space system of the mesophyll of *Poa* leaves during the uredial and telial stages of this rust (Fig. 5.3). Unlike hyphae of the monokaryon in *Tussilago*, dikaryotic hyphae do not grow within the cell walls of *Poa* and generally are in less close contact with host cells than those of the haploid mycelium (Fig. 5.4). This is confirmed by S.E.M. examination, where the monokaryotic hyphae (Fig. 5.1) appear more closely appressed to the host cells than those of the dikaryon in *Poa* (Figs. 5.3, 5.5). Amorphous material, possibly adhesive in function, is frequently deposited between

monokaryotic hyphae and host cells (Fig. 5.6) but very little of this is associated with the dikaryotic hyphae in *Poa*.

In both hosts, intercellular hyphae possess a well-developed endoplasmic reticulum, mitochondria, abundant lipid drops and vacuoles, which frequently contain vesicles with a membranous inclusion (Figs. 5.7, 5.8). In infected tissue of *Tussilago* stained with lead citrate, aggregates of electron-lucent areas of granular appearance, unlike that of either lipid drops or vesicles, suggest the presence of glycogen throughout the monokaryotic thallus in *Tussilago* (Figs. 5.9, 5.10, 5.26) but further investigation is required to confirm this. No such appearance has been found in similarly prepared sections of infected *Poa* leaves and glycogen appears to be absent from the dikaryon in *Poa*. The nuclei of both monokaryotic and dikaryotic hyphae are normally spherical or oval and surrounded by a double membrane. Dikaryotic nuclei show a somewhat greater proportion of heterochromatin relative to euchromatin than monokaryotic nuclei; they also appear to differ in that none of the sections of nuclei from monokaryotic cells examined has shown a nucleolus, whereas most of those from dikaryotic cells include a nucleolus (Figs. 5.7, 5.8, 5.10, 5.11).

Crystal-containing microbodies are observed in both monokaryotic and dikaryotic hyphae. They are frequently in the vicinity of the septal pore apparatus (e.g. Figs. 5.14, 5.15a,b), but are also seen dispersed in the cytoplasm (Fig. 5.11, 5.12). In dikaryotic but not in monokaryotic hyphae, crystal-containing microbodies are occasionally associated with lipid droplets (Fig. 5.11, 5.22).

5.1.1 Hyphal septa

Both mono- and dikaryotic hyphae of *Puccinia poarum* display a similar range of septal types:

i) Perforate septa, which develop centripetally from the longitudinal walls, consist of two electron-dense layers separated by an electron-lucent lamella, and tapering towards the central pore (Fig. 5.13). On either side of the pore is a characteristic pore apparatus (cf. Littlefield & Heath 1979) consisting of a dense organelle-free region of cytoplasm of homogenous granular appearance which differs from the surrounding hyaloplasm (Fig. 5.14). Crystal-containing microbodies, empty vesicles and electron-dense vesicles occur around the pore apparatus. Some electron-dense vesicles lie within the pore apparatus region (Figs. 5.15a,b). Examination of several sections from different samples indicates that the number of crystal-containing microbodies at the periphery of the septal pore apparatus varies from one to three per section (Figs. 5.15, 5.16, 5.17). Septal pores are frequently occluded by electron-dense material, which appears to be similar to the contents of the vesicles within or around the pore apparatus (Figs. 5.14, 5.18). The plasma membrane appears continuous over the septum and pore, and lomasome-like structures frequently occur between the fungal plasmalemma and the septum (Figs. 5.15b, 5.18). Perforate septa are frequent in hyphae growing in intercellular spaces of the host.

ii) Partial septa which appear to be formed by infolding of longitudinal walls of hyphae in intercellular mycelium are occasionally found within the mseophyll (Figs. 5.19, 5.20) but are particularly frequent in the pseudoparenchymatous tissue of aecial primordia and of urediosori (Fig.3.38,Ch.3). In monokaryotic hyphae, partial septa most frequently develop from one side only of the hypha (unilateral) (Fig. 5.19). Both unilateral and bilateral (Fig. 5.20) partial septa occur in dikaryotic hyphae. Partial septa are distinguished from the perforate septa by the larger diameter of the septal pore, the rounded, not tapering margin of the wall around the pore, the plasma membrane running around the margin of the septum, rather than appearing continuous over the pore, and the absence of a septal 'pore apparatus', so that cellular components pass freely between compartments. Crystal-containing microbodies are occasionally associated with the unilateral partial septa in dikaryotic hyphae (Fig. 5.21) but have not, so are, been observed in this position in monokaryotic hyphae.

iii) Complete septa, lacking a pore and pore apparatus, occur only in pseudoparenchymatous fungal tissue, both mono- and dikaryotic (Fig. 5.22). Besides the absence of a pore, which has been confirmed by serial sectioning, they are further distinguished from perforate septa by being thinner, but of uniform thickness, not tapering towards the centre, and by lacking a pore apparatus.

Table 5.1 summarizes differences between intercellular hyphae of *Puccinia poarum* on its alternate hosts and compares features of the inter- and intracellular structures of the parasite.

5.2 Intracellular structures

In *Tussilago* leaves, mesophyll cells are penetrated by relatively unmodified hyphae which differ in many ultrastructural features from the haustoria produced by the dikaryon in infected mesophyll cells of *Poa* (Table 5.1). Intracellular hyphae arise as terminal cells of intercellular hyphae (Figs.5.23,5.24,5.25). The mother cell of an intracellular hypha is separated from the rest of the intercellular mycelium by a septum (Fig. 5.26a,b). A distinct layer of amorphous material surrounding the hyphae and particularly noticeable between the hypha and the host cell wall, appears to play a part in the attachment of the intracellular hypha mother cell to the host cell (Fig. 5.26a). There is little such material associated with the haustorium-mother cell in *Poa* (Fig. 5.27). At the point of contact with the host cells, the wall of a monokaryotic hypha is of uniform thickness and density (Fig. 5.26a,b). The intracellular hypha mother cell protrudes to form a penetration peg, in which the continuity of the fungal wall is unbroken, and host wall-like material is deposited in advance of the penetrating hypha (Fig. 5.26a). The hypha is relatively little constricted where it penetrates the host cell wall (Fig. 5.26a,b). These features contrast with the localized thickening of the wall of the haustorium mother cell adjacent to a mesophyll cell of *Poa*, the abrupt interruption of the

fungal wall where the haustorium passes through the host cell wall (Fig. 5.27), the extremely constricted haustorial neck and its distinctive neck band (Fig. 5.28a,b). It appears unlikely that any thin layer of fungal wall material surrounds the plasmalemma of the haustorial neck in the region where it passes through the host cell wall (Fig. 5.27).

The filamentous intracellular hyphae of the monokaryon grow in an irregular manner (Figs. 5.24, 5.25). Light and electron microscopy reveal that intracellular hyphae of *Puccinia poarum* end in the host cells and are not observed to emerge from them. The septa, occasionally observed in EM sections of the intracellular hyphae (Fig. 5.29), appear similar in structure to those of the intercellular mycelium. Examination with the light microscope of infected host cells of comparable age in semi-thin sections (0.5-1.0 µm thick) shows that the frequency of occurrence of septate intracellular hyphae is higher than suggested by electron micrographs. The cytoplasm of the intracellular hypha with its nucleus, mitochondria, endoplasmic reticulum, vesicles and vacuoles is bounded by a well-defined plasma membrane (Fig. 5.30a,b). In comparison with the uredial haustoria, more lipid bodies are observed in intracellular hyphae of the monokaryon in *Tussilago* (Figs. 5.29, 5.30b).

In contrast to the monokaryotic intracellular structures, the haustorium of the dikaryotic thallus is of a well-defined form, consisting of a narrow tubular neck region, which extends into the host cell and expands to form the haustorial body (Fig. 5.28a,b). An electron-dense neckband is visible near the mid-point of the haustorial neck (Fig. 5.28a). The fungal plasmalemma is continuous between the

mother cell and the haustorium and no intervening septum is formed (Fig. 5.27). However, a septum separating the haustorium mother cell from the rest of the intercellular hypha is shown in Fig. 5.31 (see also Fig. 4.10, Chapter 4). The haustorium contains a well-developed endoplasmic reticulum, numerous mitochondria, vesicles, vacuoles and lipid drops (Fig. 5.32a,b).

Compared with the intercellular hyphae of the dikaryon, uredial haustoria appear to have smaller amounts of lipid drops (see Figs. 5.4, 5.8, 5.32a,b). In all haustoria of *P. poarum* so far examined, by light- or electron-microscopy, only a single nucleus has been observed in the body of the haustorium (Figs. 5.32b,c; see also Fig. 4.13, Chapter 4) although in Fig. 5.27, one nucleus is observed in the narrow neck region. The well-defined plasmalemma of the haustorium body is occasionally invaginated by lomasomes (Fig. 5.33). In older haustoria, the mother cell becomes highly vacuolated (Fig. 5.27).

The effect on the host wall of penetration by the parasite differs considerably in the two hosts. In *Tussilago*, the intracellular hypha mother cell forms a penetration peg, projecting into the host cell, encased in a thick layer of host cell wall material (Fig. 5.26a). Penetration of *Poa* cells by the dikaryon involves little disturbance or change in the region of the host cell wall penetrated by the haustorium (Fig. 5.27). There is no collar of host wall-like material around necks of haustoria of the dikaryotic thallus of *P. poarum* whereas this appears to be a normal feature of the intracellular hyphae of the monokaryon (Fig. 5.34), clearly corresponding to the layer of host wall-like material formed around

every penetration peg (Fig. 5.26a). By further growth the intracellular hypha penetrates this wall layer and grows within the cell, enclosed by the host plasmalemma.

Sections of infected leaf tissue stained with saturated recorcin blue (lacmoid) in 30% ethyl alcohol (see Appendix) showed a blue, callose type of staining on intracellular hyphae and on the host wall adjacent to the site of penetration. No such reactions were associated with haustoria of the dikaryon nor with either monokaryotic or dikaryotic intercellular mycelium. In both hosts, whether infected or not, positive callose reactions are given by vascular tissue of the leaves. In *Poa*, there was a particularly clear reaction by the walls of both xylem and phloem, endodermis and the outer walls of epidermal cells. The staining of infected and uninfected tissues was compared with that of *Vitis* phloem which is known to contain callose (Currier 1957). In woody stems of *Vitis*, tissues other than phloem fail to show a callose reaction.

The distribution of fluorescence observed in leaf tissue stained with analine blue (see Appendix) confirms these observations. In this procedure, unstained tissue is also examined under the fluorescence microscope in order to determine the primary fluorescence of the tissue. Infected leaf tissue of *Tussilago* stained with analine blue shows a strong yellow fluorescence along intracellular hyphae of *P. poarum* and also in the host cell wall around the point of penetration. No fluorescence is shown by the monokaryotic intercellular mycelium. Infected *Poa* leaves show no differences in distribution of fluorescence between infected and uninfected leaf tissue and no association of fluorescence with haustoria or with

dikaryotic intercellular hyphae. Fluorescence of the vascular tissue of both hosts corresponded with the distribution of callose indicated by the resorcinol blue method.

Both intracellular hypha and haustorium are surrounded by a matrix (corresponding to the term 'extrahaustorial matrix' suggested by Bushnell 1972), which is bounded by the invaginated host plasma membrane (Figs. 5.30b, 5.32, 5.34, 5.35). A frequent general feature of this region in the monokaryotic intracellular hypha is the deposition of variable amounts of material closely resembling the host cell wall, directly on the more electron-dense fungal wall (Figs. 5.34, 5.36). Any space between this deposit and the bounding membrane of invaginated host plasmalemma usually contains fibrillar material of similar electron density (Figs. 5.30a, 5.36). In addition, the plasma membrane surrounding the intracellular hypha is observed to form vesicle-like structures enclosing membranous materials (Fig. 5.37) or electron-dense granules (Figs. 5.38a,b). Moreover, vesicles are observed within the matrix and around its bounding membrane (Fig. 5.38c). The deposition of material resembling the host cell wall is not detected in uredial haustoria. Instead, the matrix around the haustorium tends to be occupied by fibrillar material of an electron density similar to that of the fungal wall and continuous with the haustorium wall material (Fig. 5.35).

In both hosts of *P. poarum*, the plasma membrane (invaginated host plasma membrane) surrounding the matrix around the intracellular structure appears to be identical in electron density and thickness to the non-invaginated host plasma membrane (Figs. 5.27, 5.29).

The haustorium is very closely associated with the host cell nucleus, which is frequently bent around it or deeply invaginated by it, separated only by a thin layer of host cytoplasm which includes metochondria and microbodies (Fig. 5.39a). The nuclei of haustorium and host cell might thus be frequently brought into close proximity (Fig. 5.40). On the other hand, neither light-microscope nor electron-microscope observations show any special association of host nucleus and intracellular hyphae in infected *Tussilago* cells, although the host nucleus occasionally lies close to an intracellular hypha as in Fig. 7.9. The chloroplasts, mitochrondria and endoplasmic reticulum of the host cell, however, are frequently found to be associated with the intracellular hyphae (Figs. 5.30b, 5.34).

A recurring feature of both haustoria and intracellular hyphae is the formation of concentric aggregations of membranes similar to those of the endoplasmic reticulum (Figs. 5.30a,b, 5.2a). In intracellular hyphae, some of the cristae of mitochondria show an obvious continuity with membranes of the endoplasmic reticulum (Fig. 5.41).

Table 5.1 and Fig. 5.42 summarize the differences between the intracellular structures produced by *P. poarum* on its alternate hosts.

Table 5.1 Comparison of intercellular and intracellular structures of *Puccinia poarum* on its alternate hosts.

	Monokaryon on *Tussilago farfara*		Dikaryon on *Poa pratensis*	
	Intercellular hyphae	Intracellular hyphae	Intercellular hyphae	Haustoria
Nucleolus visible	-	-	+	-
Glycogen-like particles	+	+	-	-
Crystal-containing microbodies	+	-	+	-
Growth in wall and middle lamella	+	-	-	-
Septation	+	+	+	-
Adhesive material between fungal and host cells	Thick layer	-	Thin layer	-
Growth within vascular bundle	+	+	-	-
Constricted neck and neckband	-	-	-	+
Collar	-	+	-	-
Localized thickening at point of penetration	-	-	-	+
Association with host cell nucleus	-	occasional	-	frequent
Contents of matrix region	-	resemble host-wall	-	electron-dense fibrils resembling fungal wall

All fungal structures contained endoplasmic reticulum, mitochondria, lipid bodies and vacuoles, which frequently included vesicles with membranous material.

CHAPTER 6

INVASION OF VASCULAR BUNDLES

6

INVASION OF VASCULAR BUNDLES

6.1 Light microscopy

In infected regions of *Tussilago* leaves, inter- and intracellular fungal structures of the monokaryon occur in the vascular bundles (Figs. 6.1, 6.2) particularly in the phloem region, in xylem parenchyma and in the bundle sheath cells (Figs. 6.1, 6.2, 6.3). Intracellular hyphae show dense growth in the vascular tissue, in striking contrast to their relatively sparse distribution in the much larger cells of the adjacent parts of the mesophyll tissue, where the bulk of the monokaryotic thallus is confined to the intercellular spaces (Fig.6.1). In earlier stages of development of the fungus, invasion of the xylem tracheary elements is not observed but at later phases of infection, where opened aecia are present on the lower surface of the leaf, intracellular hyphae appear to occur also in the xylem vessels. The presence of intracellular hyphae in the xylem vessels of *Tussilago* leaves has been confirmed by serial sections passing through this region (Fig. 6.4a,b).

In *Poa* leaves where xylem and phloem are enclosed by two layers, the sheath of large, longitudinally elongated cells and the heavily thickened endodermis, intracellular fungal structures of the dikaryon are seen in the bundle sheath cells (Fig. 6.5) but not in cells of the endodermis or vascular tissue within this layer. These bundle

sheath haustoria tend to be elongated when viewed in longitudinal section (Fig. 6.6).

6.2 Electron microscopy

6.2.1 *Tussilago farfara*

The vascular bundles of *Tussilago* leaves are surrounded by a layer of compactly arranged parenchymatous cells, the bundle sheath (or border parenchyma, Esau 1965). Within this layer lies the phloem, with its companion cells, phloem parenchyma, transfer cells and sieve tubes, and the xylem, with its parenchyma cells and tracheary elements. In infected *Tussilago* leaves, invasion of the vascular bundles by fungal hyphae is found at all stages of development of pycnial-aecial infection. Fig. 6.7 shows a small vascular bundle in a young pycnial pustule (P1 stage, 4 mm diameter, aecia not visible). Intercellular hyphae occur in almost every intercellular space between bundle sheath and mesophyll. Intracellular hyphae are also present in bundle sheath cells and in the living cells associated with the xylem and phloem. Also, in young pycnial pustules, fungal structures occur similarly in larger vascular bundles (Fig. 6.8). Even more extensive penetration of vascular tissue occurs in larger veins at later stages of infection (P2) (Fig. 6.9), when aecia are forming on the lower surface in areas about 6 mm in diameter. At this time, almost all living cells of the vascular bundle contain fungal structures. The more dense growth of the monokaryotic hyphae in the vascular bundle than in adjacent

regions of the mesophyll, indicated by light microscopy, has been confirmed by electron microscopy. Intracellular hyphae are more frequent in the bundle sheath cells than in the living cells associated with xylem or phloem (Figs. 6.7, 6.9).

As in certain other members of the Compositae (Browning & Gunning 1977), the phloem of *T. farfara* leaves is characterized by the development of wall ingrowths of the transfer cell type in phloem parenchyma and the companion cells of sieve tubes (Fig. 6.10). Most of these cells are penetrated at least by one intracellular hypha and have rather electron-dense cytoplasm with an enlarged, lobed nucleus (Figs. 6.10, 6.11). In healthy phloem an intervening space is observed between the wall ingrowths and the plasmalemma but no such space exists in the transfer cells of the infected phloem (Figs. 6.10, 6.11, 6.12). However, lomasome-like structures occasionally occur between the plasmalemma and wall ingrowths in the transfer cells of the infected phloem (Figs. 6.11, 6.13).

Almost all living cells associated with the phloem of healthy vascular bundles have wall ingrowths (Figs. 6.12, 6.14). The frequency of such transfer type cells and the complexity of their wall proliferations appears lower in the infected phloem region than in the healthy phloem of comparable age (compare Figs. 6.7, 6.8, 6.9, 6.12, 6.14).

In healthy and infected leaves of *Tussilago*, no transfer cell ingrowths occur in xylem parenchyma. Compared with phloem parenchyma, xylem parenchyma cells show a higher degree of vacuolation and less dense cytoplasm but, like the phloem parenchyma, are extensively invaded by intracellular hyphae (Figs. 6.7, 6.8, 6.9, 6.15a,b).

Hyphae which occur between the cells of the xylem and phloem (Figs. 6.9, 6.15, 6.16a) or project into the cell lumen of bundle sheath cells (Figs. 6.15a,b, 6.16b) are commonly surrounded by a material continuous with and similar to the host cell wall. Around intracellular hyphae, this material is often irregular in thickness where the hyphae may have been close to their point of entry. Although the presence of intracellular hyphae in the tracheary elements is indicated by light microscopy, ultrastructural examination has so far failed to find fungal structures in these elements but hyphae often lie in close contact with them (Figs. 6.7, 6.15b), separated from the vessel lumen only by a thin layer of host wall (Fig. 6.16a). Figs. 6.15 and 6.16b show sign of breakdown of walls of xylem elements similar to that described by Harder (1978). The infected vascular bundles appear similar in structure to those of healthy parts from infected leaves but differ in cytological detail. Nuclei of healthy phloem parenchyma are of the normal oval form (Fig. 6.14) whereas those of infected calls are enlarged and variable in shape (Fig.6.19b). Nucleoli are frequently present in the nuclei of the healthy phloem parenchyma (Figs. 6.14, 6.17) but not in the nuclei of the infected phloem parenchyma cells (Figs. 6.10, 6.18).

The generally transverse view of intracellular hyphae seen in transverse sections of vascular tissue and bundle sheath cells indicates their longitudinal path in the vein. This in in striking contrast to the intercellular and intracellular hyphae of the mesophyll, which lack any uniform orientation. The previous light microscope observations, mentioned earlier in this chapter, also suggest that intracellular hyphae make extensive growth in the vascular bundles parallel to the conducting elements.

The intracellular hyphae of vascular bundles appear metabolically active, having dense cytoplasm, prominent nuclei and mitochondria, lipid and glycogen-like particles, and each is enclosed by a clearly-defined, membrane-bound layer, resembling the extrahaustorial sheath of dikaryotic growth stages (Harder 1978) for which Littlefield & Heath (1979) have used the term extrahaustorial matrix, suggested by Bushnell (1972). The walls of such intracellular hyphae vary in thickness and electron density but are generally less dense than those in bundle sheath cells and those of intercellular hyphae.

Mature intracellular hyphae of bundle sheath cells have a thick, electron-dense wall layer (Fig. 6.10, 6.19a), surrounded by a layer of material with a similar staining reaction to the host wall and to the material in which hyphae lying adjacent to the cell wall of bundle sheath cells (Figs. 6.9, 6.15a, 6.19a, 6.20) are embedded. Younger intracellular hyphae of bundle sheath cells and those in phloem and xylem parenchyma (Figs. 6.9, 6.10, 6.19b) have a thinner, less electron dense wall layer separated from the external membrane by a transparent region which, in some cases, appears to be distended and to contain fibrillar material (Fig. 6.19b). In contrast, as already noted, the wall ingrowths of the transfer cells in infected vascular bundles are closely surrounded by plasmalemma with no visible intervening space.

6.2.2 *Poa pratensis*

Both scanning and transmission electron microscopes have been used to investigate the question of the infection of vascular bundles

of *Poa* leaves. Similar to previous light microscopy, the transmission/microscopic examination reveals no invasion of endodermis, phloem and xylem by the parasite. Although, at the light microscope level, haustoria have been observed in bundle sheath cells, no fungal intracellular structures of the dikaryon are observed in these cells using transmission electron microscopy. However, the scanning electron microscope shows that uredial haustoria occasionally occur in the bundle sheath cells (Fig. 6.21). In Figs.6.22 and 6.23, intercellular hyphae are seen growing in the intercellular spaces between the bundle sheath and the adjacent mesophyll. Neither light nor electron microscope has shown invasion by the parasite of fibers associated with the vascular bundles.

Ultrastructurally, no differences are observed between vascular bundles from healthy and infected parts of *Poa* leaves. The phloem parenchyma and companion cells show nuclei, mitochondria, endoplasmic reticulum and lipid drops (Figs. 6.24, 6.25a,b). These cells are generally rich in free ribosomes (Fig. 6.26). An electron-dense body with double-membrane is frequently observed in phloem parenchyma cells (Figs. 6.25a, 6.26). Plastids are also seen in both phloem parenchyma and companion cells (Fig. 6.25a,b). Fig. 6.27 shows young sieve tubes with mitochondria and vacuoles. The protoplasts of phloem cells of *Poa* leaves are connected by separate unbranched plasmadesmata (Fig. 6.25a,b) while, in *Tussilago*, these structures are dichotomously branched and more frequent than in *Poa*. Fig. 6.28 shows electron-transparent areas around plasmadesmata connecting two adjacent sieve elements. These areas may be sites of callose deposition.

Xylem parenchyma cells show nuclei, mitochrondria, endoplasmic reticulum, vacuoles and large amounts of ribosomes (Figs. 6.29a,b, 6.30, 6.31). Fig. 6.31 shows xylem elements with lignified thickenings and remnants of the cellulosic primary walls. No transfer cells are observed in vascular bundles of either healthy or infected *Poa* leaves.

The endodermis in *Poa* leaves consists of cells heavily thickened on their inner and radial walls, and is connected to the abaxial epidermis by other fibres (Figs. 6.5, 6.32). Fig. 6.32 shows a crystal-containing microbody and cytoplasm in an endodermal cell but although they are living cells and only thickened on inner and radial walls they are not penetrated by haustoria and clearly form a barrier to invasion of the vascular system. The bundle sheath cells appear similar in cytology to those of the mesophyll but small in size (Fig. 6.33) and less frequently penetrated by haustoria, which although seen occasionally in light microscope preparations, have so far escaped detection with the electron microscope.

CHAPTER 7

CHANGES IN TISSUES OF ALTERNATE HOSTS

7

CHANGES IN TISSUES OF ALTERNATE HOSTS

7.1 Light microscopy

7.1.1 Host cell size

In longitudinal sections through the mesophyll tissue of *Tussilago* leaves, parallel to epidermis, marked differences in size are observed between mesophyll cells in the vicinity of pycnia and those further away (Fig. 7.1). Measurements recorded in Table 7.1 demonstrate that host cells adjacent to a pycnial pustule are significantly smaller than those at a distance from it or those in uninfected mesophyll tissue. No such effect of infection has been observed in *Poa*.

7.1.2 Host nuclei

No particular association of nuclei of infected cells of *Tussilago* with intracellular hyphae is observed nor any striking changes in shape, when compared with the nuclei of uninfected cells. However, in both mesophyll and epidermal cells of infected parts of a *Tussilago* leaf, enlargement of the host nuclei is found. The volume of *Tussilago* mesophyll nuclei increases by 53%, about half as much as in nuclei of infected epidermal cells, which show an increase in nuclear size of 100% (Table 7.2).

Table 7.1 Comparison of areas of mesophyll cells of *Tussilago farfara* adjacent to and at some distance from pycnium, as seen in sections parallel to upper epidermis.

	Cell adjacent to pycnium	Cell >100 µm distant from pycnium
Host cell area (μm^2)	1442.6±101.1	2149.3±240.9
Number of cells measured	45	32

± Standard error, 10 pycnia from several pycnial-aecial postules (P3).

Table 7.2 Response of host cell nucleus to infection by *Puccinia poarum*.

	Volume (μm^3) of nucleus†					
	*Tussilago farfara**			*Poa pratensis***		
	Healthy	Infected	Increase %	Healthy	Infected	Increase %
Mesophyll	102.6 ± 3.6	157.1 ± 7.0	53.1	93.1 ± 5.3	194.7 ± 11.8	109.1
Epidermis	101.7 ± 3.7	215.9 ± 11.4	112.3	107.6 ± 4.8	149.0 ± 7.2	38.5

† Means of 30 nuclei ± standard error.

* Pycnial-aecial pustule (>50% of aecia dehisced, i.e. P3).

** Uredial-telial pustule (24 days after inoculation).

The nucleus of infected cells in *Poa* leaves generally lies adjacent to the fungal haustorium (Fig. 7.2), often bent closely around it. Host cell nuclei are noticeably larger than those of healthy cells (Table 7.2) (Fig. 7.3a,b), their volumes being increased by 109% in mesophyll and 39% in epidermal cells after penetration by haustoria. Although multiple infections of epidermal cells are observed, the epidermal cell nucleus appears less affected than those in infected mesophyll cells. Nuclei of infected cells are also more variable in shape, frequently rather elongated compared with the approximately spherical nuclei of healthy mesophyll cells. Greatest elongation of the nucleus is seen in infected epidermal cells.

7.1.3 Host chloroplasts

Comparison at the light microscope level, using resin-embedded sections, indicates that infected mesophyll cells of *Tussilago* leaves contain fewer chloroplasts than adjacent uninfected cells (Fig.7.4a,b). In Table 7.3, in discs of infected and uninfected leaf tissue from leaves of comparable age, the infected *Tussilago* mesophyll cells during P1 (pycnial pustule, aecia not visible) and P2 (<50% of aecia dehisced) stages of pycnial infections show a significant reduction in chloroplast numbers, compared with the uninfected cells. Similar reduction has been found in mesophyll cells from several pycnial pustules at very early stages (1-2 mm diameter) although the number of chloroplasts in comparable uninfected cells appears to increase with age (Fig. 7.5a-f). In older uninfected mesophyll cells, one

to three starch grains occur in the chloroplasts (Fig. 7.6a). Starch grains are also present but fewer, in comparable infected cells (Fig. 7.6b).

The characteristic yellowing of infected sites on leaves of *Tussilago* during the development of the rust infection is correlated with changes in colour and appearance of the chloroplasts, as well as their reduced number compared with uninfected regions of the leaves (Table 7.3).

Association of the host chloroplasts with the monokaryotic intracellular hyphae is frequently observed, where the chloroplast and cytoplasm appear to be pulled away from the host cell wall (Fig. 7.4b).

Using similar, one micron-thick, resin-embedded sections, the number of chloroplasts in both infected and uninfected mesophyll cells of *Poa* has been studied. Chloroplast numbers are significantly reduced in mesophyll cells after infection by the dikaryon, when compared with uninfected cells (Table 7.3) (Fig. 7.7a,b). Table 7.3 shows that the infected mesophyll cells lose about 40% of their chloroplasts during uredial infection (10-14 days from inoculation). Moreover changes are observed in the green colour of the chloroplasts in infected cells (Table 7.3). Freezing microtome sections of both young and old healthy leaves of *Poa* show that the number of chloroplasts per cell increased with age (compare Fig.7.7 b and c).

Table 7.3 Responses of host chloroplasts to infection by *Puccinia poarum* observed under light microscope. In both hosts, differences between infected and uninfected cells exceed the 0.05 level of significance.

	Tussilago farfara			*Poa pratensis*		
	Uninfected cell	Infected cell		Uninfected cell	Infected cell	
	HC	P1	P2	HC	U1	U2
Mean number of chloroplasts* in cell	12.7 ± 0.4	6.7 ± 0.2	5.9 ± 0.2	9.7 ± 0.3	5.9 ± 0.3	5.7 ± 0.2
% loss in chloroplasts		47.3	53.5		39.2	41.2
Colour of chloroplasts**	green	yellowish green	yellowish green	green	yellowish green	yellowish green

* Counts of 100 cells (± standard error of means) in 25 slides from 5 specimens of comparable age, each sample consisting of cells lying on the ends of a transect line in section through mesophyll tissue parallel to the leaf surface.

** Detected in freezing microtome sections.

HC: Healthy cell from infected leaf

7.2 Electron microscopy

7.2.1 Host nucleus

In *Tussilago*, the nucleus of the uninfected mesophyll cells (Fig. 7.8a-c) is bounded by a well-defined double membrane and shows clear differentiation of heterochromatin and euchromatin. The heterochromatin is concentrated in the periphery of the nucleus and its amount appears to be less than that of the euchromatin. A nucleolus is also present in the nucleus of uninfected cells (Fig. 7.8b,c).

The mesophyll cell nucleus is not always close to an intracellular hyphae in infected *Tussilago*, where such association is only observed during later stages of infection (Fig. 7.9). The nuclei appear to be elongated compared to normal nuclei of uninfected tissue (compare Figs. 7.8b and 7.10a). As in the normal nucleus, heterochromatin aggregates in the periphery of the nucleus in infected *Tussilago* cells (Fig. 7.10a,b) but, as infection progresses, the amount of heterochromatin appears to be less than that of the normal nucleus (Figs. 7.9, 7.11). Figs. 7.9 and 7.11 show very small clumps of heterochromatin in the nucleus of *Tussilago* mesophyll cells during later stages of infection.

In nuclei of infected mesophyll cells, the electron-density of euchromatin is increased, compared with nuclei of uninfected cells (Figs. 7.8a,b,c, 7.9, 7.10a,b).

In infected mesophyll cells of *Tussilago*, the nucleolus is associated with electron-dense material which appears to originate

from it (Figs. 7.10, 7.11). Nucleoli are occasionally seen during later stages of infection.

In mesophyll cells from uninfected parts of *Poa* leaves, the nuclei are oval or spherical and show large amounts of heterochromatic material, nucleoli and double unit nuclear membranes (Fig. 7.12a, b,c). Those of older healthy mesophyll cells show nucleoli and a balanced ratio of euchromatin and heterochromatin (Fig. 7.13).

In infected mesophyll tissue of *Poa*, the fungal haustorium is closely associated with the host cell nucleus which is frequently bent around or deeply invaginated by it (Figs. 7.14a,b, 7.15, 7.16a, b). The section shown in Fig. 7.16a and b shows a haustorium completely surrounded by the host nucleus during later stages of infection. A marked increase in size of the host nucleus (recorded in Table 7.2), an irregular, lobed form and changes in the proportion and density of euchromatin occur in mesophyll cells containing haustoria. More euchromatin than heterochromatin is found in nuclei of infected cells during both early (Fig. 7.14a,b) and later stages (Figs. 7.15, 7.16a,b, 7.17) of uredial infection. In addition the electron-density of the euchromatin also increases during the development of the uredial and telial stages (Fig. 7.14b, 7.16a,b, 7.17). Compared with nuclei of uninfected cells, the amount of heterochromatin decreases in those of infected mesophyll cells (Figs. 7.13b, 7.14a,b, 7.15, 7.16a,b). After infection, nucleoli appear to be absent in nuclei of mesophyll cells of *Poa*, although very occasional sections of nuclei during early stages of infection show nucleoli (Fig. 7.14b). However, fewer nucleoli are observed in mesophyll cells of infected *Poa* than in infected *Tussilago*.

Table 7.4 summarizes the ultrastructural changes in nuclei of *Poa* and *Tussilago* mesophyll cells after infection by *Puccinia poarum*.

7.2.2 Host chloroplasts

Compared with the uninfected tissue, changes are observed in the ultrastructure of chloroplasts in infected *Tussilago* mesophyll cells (Table 7.5). Chloroplasts from uninfected tissue contain a well-developed membrane structure of grana, intergranal lamellae, and chloroplast-bounding membranes with few osmiophilic globules (Fig. 7.18). In contrast, chloroplasts of comparable tissue during early stages of pycnial-aecial infection show elongation of intergranal lamellae, reduction in number and size of grana, increase in number and size of lipid drops and the appearance of starch grains (Fig. 7.19). The later stages of infection in *Tussilago* are accompanied by degeneration of the chloroplast with the breakdown of grana, intergranal lamellae and the chloroplast envelope (Fig.7.20a-d). By this stage, degradation of starch grains and osmiophilic globules has occurred (Fig. 7.20c,d). The host chloroplasts are frequently in close proximity to fungal intracellular hyphae (Figs. 7.20a, 7.21), an association which is one of the important features distinguishing intracellular hyphae of the monokaryon from uredial haustoria.

In aged healthy cells of *Tussilago* the chloroplasts are elongated, containing a well-developed membrane structure with very dense stacks of grana and one to three large starch grains (Fig. 7.22a,b). In these chloroplasts, osmiophilic globules are very few or absent.

Table 7.4 Ultrastructural changes in nuclei of alternate hosts during infection by *Puccinia poarum*.

	Pycnial-aecial infection on *Tussilago farfara*		Uredial-telial infection on *Poa pratensis*	
	P1	P2	U1	U2
Nucleus				
Association with intracellular structure of parasite	Absent	Occasional	Always closely associated with haustoria	
Shape	Elongated, enlarged	Elongated, enlarged	Enlarged, irregular, slightly curved around haustorium	Enlarged, very irregular, invaginated by haustorium and elongated
Nucleolus	Normal	Associated with electron-dense material	Occasionally present	Absent
Chromatin material	Heterochromatin less than euthromatin and concentrated in periphery of nucleus	Euchromatin more electron-dense	Heterochromatin less in amount than euchromatin	Euchromatin more electron-dense

Table 7.5 Ultrastructural changes in chloroplasts of alternate hosts during infection by *Puccinia poarum*.

	Pycnial-aecial infection on *Tussilago farfara*		Uredial-telial infection on *Poa pratensis*	
	P1	P2	U1	U2
Chloroplasts				
Grana	Reduced in size	Degenerated	Normal	Occasionally swollen
Integranal lamellae	Elongated	Not visible	Normal	Occasionally swollen
Envelope membrane	Normal	Degenerated	Normal	Occasionally encloses vesiculated area, frequently associated with Golgi bodies, R.E.R. and micro-bodies
Starch grains and osmiophilic globules	Increased in size and number	Degraded	Increased in size and number	Further increased
Association with fungal intracellular structures	Present	Present	Absent	Absent

The fine structure of chloroplasts from uninfected mesophyll tissue of *Poa* shows well-developed grana, intergranal lamellae and bounding membranes with few osmiophilic globules (Fig. 7.23). In older healthy cells, the chloroplasts contain an extensively developed membrane structure with 1-2 large starch grains while osmiophilic globules are either absent or very few (Figs. 7.13, 7.24). In these chloroplasts vesiculated areas were also observed (Fig. 7.25).

During uredial and telial development in *Poa* mesophyll tissue, the chloroplast membrane structure (Fig. 7.26) resembles that in uninfected cells. An increase in number and size of the osmiophilic globules and the appearance of starch grains are detected in the chloroplast during early stages of infection (Figs. 7.26, 7.27), a trend which continues into later stages of uredial and telial development (Fig. 7.28a,b), where Golgi bodies, rough endoplasmic reticulum and microbodies are frequently associated with the chloroplast envelope. In the early stages of uredial infection, swollen grana and intergranal lamellae and peripheral vesicles are occasionally seen in the chloroplasts (Figs. 7.29, 7.30). Similar swelling of grana and intergranal lamellae is occasionally observed in chloroplasts of *Tussilago* during early stages of pycnial infection (Fig. 7.31).

In contrast to *Tussilago* cells infected by monokaryotic intracellular hyphae, there appears to be no special association of the host chloroplasts with intracellular structures formed by uredial-telial infections in *Poa*. Table 7.5 summarizes the ultrastructural changes in chloroplasts of the alternate hosts of *Puccinia poarum*.

7.2.3 Host microbodies

As in other higher plants (Breidenbach 1976; Beevers 1979), microbodies occur in both infected and uninfected mesophyll cells of *Tussilago*. These microbodies are bounded by a single membrane and contain granular material and frequently a large crystal body. In infected mesophyll cells, during P2 stage of rust development in *Tussilago* (<50% of aecia dehisced), crystal-containing microbodies are found close to the chloroplast, nucleus, mitochondria, and host cell wall (Fig. 7.32a,b,c). A similar association occurs in comparable cells from uninfected areas (Figs. 7.8a, 7.33). No microbodies are detected in infected mesophyll cells during later stages of monokaryotic infection. Microbodies in corresponding uninfected cells generally lack crystals, although traces of crystal (Fig.7.34) are found in some. The morphology of the crystals is, however, variable in both infected and uninfected mesophyll cells of *Tussilago*.

When compared with uninfected cells, frequency of crystal-containing microbodies in infected *Tussilago* mesophyll cells is remarkably decreased (Table 7.6), the number per cell section being reduced by half by the time aecia are opening.

In *Poa* mesophyll cells, the microbodies show a similar appearance to that observed in *Tussilago*, but fibrillar units, resembling those reported by Fredrick & Newcomb (1971), are observed in some crystal-containing microbodies of uninfected *Poa* mesophyll cells (Fig. 7.35a,b). In both infected and uninfected cells, variations occur in the size and morphology of crystals and the amount of granular material present in microbodies (Figs. 7.35a,b,c, 7.36, 7.37, 7.38).

Table 7.6 Responses of host crystal-containing microbodies to infection by *Puccinia poarum*.

	Tussilago farfara			*Poa pratensis*		
	HD	P2	P3	HD	U2	U3
Microbodies per cell profile						
Occurrence	+	+	-	+	+	+
Number/cell section	1.9 ± 0.3	0.8 ± 0.2	-	4.0 ± 0.3	2.6 ± 0.3	a
Association with host wall	+	+	-	+	+	+
Association with other host organelles	With chloroplast, mitochondria and nucleus		-	With mitochondria, chloroplast, nucleus		Frequently with chloroplast, and occasionally with Golgi bodies

Counts of 20 cells from 20 resin-embedded ultra-thin sections of several specimens of comparable ages.

± Standard error of means; a, not counted; + = present; - = absent.

In comparison with uninfected cells, the number of crystal-containing microbodies per cell section is significantly decreased in infected mesophyll cells of *Poa* (Table 7.6). In contrast to *Tussilago*, crystal-containing microbodies are observed in mesophyll cells of *Poa* during both early (Fig. 7.39) and later stages (Fig. 7.41) of uredial infection. These microbodies are not seen in older healthy mesophyll cells of *Poa*. Crystal-containing microbodies are frequently associated with other host organelles (Figs. 7.35, 7.37, 7.38) particularly with chloroplasts (Figs. 7.35a,b, 7.36) in both infected and uninfected cells. Sections frequently show an association of more than one microbody with chloroplasts of the infected cells during later stages of infection (Figs. 7.40, 7.41).

Responses of host microbodies to infection by *Puccinia poarum* are recorded in Table 7.6. More crystal-containing microbodies are observed in infected and uninfected mesophyll cells of *Poa* than in those of *Tussilago*.

7.2.4 Other host organelles

Mitochondria in infected cells of *Tussilago* tend to lie in close association with intracellular structures of the monokaryon and show vesiculation and degeneration during later stages of pycnial-aecial infections in *Tussilago* (Figs. 7.20b,d, 7.42). Such vesiculation of host mitochondria is not seen in uninfected mesophyll cells. In infected mesophyll cells of *Poa*, the host mitochondria appear unchanged, compared to those of the uninfected cells, although they also are associated with the fungal intracellular structures of the dikaryon (Figs. 7.12c, 7.16b, 7.45a).

Golgi bodies have not been detected in either infected or uninfected cells of *Tussilago*. In uninfected mesophyll cells of *Poa*, Golgi bodies are found adjacent to the host cell wall (Fig. 7.43), while those in infected cells frequently lie close to a chloroplast (Figs. 7.44a,b, 7.45). Golgi bodies of the infected mesophyll cells appear to increase in number during later stages of infection (Fig. 7.44b) and are occasionally seen to be associated with other host organelles such as mitochondria (Fig. 7.45a) and microbodies (Fig. 7.45b).

After penetration of mesophyll cells by uredial haustoria, the host endoplasmic reticulum is frequently observed near haustoria. Fig. 5.32b shows cisternae of endoplasmic reticulum closely associated with the invaginated plasma membrane (extrahaustorial membrane). However, no continuity was observed between endoplasmic reticulum (E.R.) and the extrahaustorial membrane. Similar results were found in *Tussilago* (see Fig. 5.30). Rough E.R. was only seen in infected mesophyll cells during later stages of uredial infection (Fig.7.45a) and was frequently associated with chloroplasts (Fig. 7.46) but not with fungal haustoria. No rough E.R. was observed in either infected or uninfected mesophyll cells of *Tussilago*.

7.2.5 Host cell wall

Besides the greater disturbance of the cell wall of *Tussilago* by the parasite, the involvement of cell wall-like material in the host-parasite interface and the presence of adhesive material between host and fungal walls (see Chapter 5), another feature in

which infected mesophyll cells of *Tussilago* differ from those of *Poa* is the presence of numerous vesicles containing granular material, close to the host cell wall (Fig. 7.47). These vesicles are bounded by plasma membrane which appears to be fused with the tonoplast and often resemble lomasomes. In Fig. 7.47, the bounding plasma membrane of this structure is also in close contact with mitochondria. No similar structures have been observed in uninfected mesophyll cells of *Tussilago*.

CHAPTER 8

AUTORADIOGRAPHIC STUDIES OF THE ASSOCIATION OF *PUCCINIA POARUM* WITH ITS ALTERNATE HOSTS

8

AUTORADIOGRAPHIC STUDIES OF THE ASSOCIATION OF *PUCCINIA POARUM* WITH ITS ALTERNATE HOSTS

8.1 The effects of infection with *P. poarum* on the uptake of label from ^{3}H-glycerol by the alternate hosts

In order to compare the physiological relationships of the monokaryotic and dikaryotic growth stages of *P. poarum* with their respective hosts, an autoradiographic study of the effects of infection on the distribution of label from tritiated glycerol has been carried out at the light microscope level. The distribution of infected and uninfected tissue and the location of tissue blocks removed for sectioning and microautoradiography are indicated in Fig. 8.1. Macroautoradiographs of excised portions of infected leaves of *Tussilago* and *Poa*, which have been supplied, via the vascular system, with [1(3)(c)-^{3}H] glycerol solution, followed by a chase period in water, show an accumulation of radioactivity in areas colonised by the fungus (Figs. 8.2 and 8.3) as well as in leaf veins.

In both hosts, microautoradiographs of leaf sections of uninfected (Figs. 8.4, 8.11 and 8.19) and infected (Figs. 8.5, 8.6, 8.7 and 8.12) leaf tissues show an accumulation of radioactivity in veins, principally in the phloem and living cells associated with the vascular tissue. Most of the silver grains in uninfected mesophyll tissue of *Tussilago* are located over the thin peripheral layer of cytoplasm, particularly over the chloroplasts (Fig. 8.8a,b,c).

Infected *Tussilago* tissue shows radioactivity associated with inter- and intracellular hyphae and host cell wall, rather than with chloroplasts (Figs. 8.5, 8.6, 8.7, 8.9 and 8.10). As far as can be determined at this level of resolution, the silver grains associated with intracellular hyphae in host cells lie just outside the fungal wall, and not generally within the hyphal lumen (Figs. 8.6b, 8.9 and 8.10). In some intracellular hyphae within vascular bundles, however, hyphal contents appear to be labelled.

The greatest density of silver grains in autoradiographs of infected *Tussilago* is found over aeciospores, the hymenial cells from which they arise and the dense fungal stroma, which underlies the hymenium (Figs. 8.13 to 8.17). The cells of the aecial peridium are strongly labelled, as also is the surrounding tissue, lining the cavity in the leaf where the aecium is embedded (Fig. 8.13). The aecium shown in Fig. 8.14 may have been at a different stage of development from that in Figs. 8.16 and 8.17, at the time of feeding with ^{3}H-glycerol, and shows intense labelling of the sub-aecial stroma but less radioactivity in the hymenium and aeciospores.

Figs. 8.16 and 8.17 show a gradient of increasing density of silver grains from subaecial stroma to hymenium to aeciospores, suggesting that the aeciospores are the sink for ^{3}H-labelled substrate. The clear association of silver grains with septa separating aeciosporophores and spore initials provide evidence of involvement of glycerol or a derivative in fungal wall synthesis. The lack of silver grains over the characteristic wall thickenings at the upper end of hymenial cells near the septum may indicate that these are older, persistent structures, formed before the time of feeding

with ^{3}H-glycerol. In autoradiographs from several aecia of *P.poarum* radioactive material is found between the aeciospores (Fig. 8.16a,b). It is suggested that this may be derived from the breakdown of intercalary cells, which are formed between aeciospores.

In uninfected mesophyll tissue of *Poa*, as in *Tussilago*, most of the tritium supplied as glycerol appears in the chloroplasts (Figs. 8.18 and 8.19). The nucleus and cytoplasm are also labelled and a particularly high concentration of radioactivity is found in lipid bodies, which frequently occur in uninfected tissue in the vicinity of uredial infections.

Infected mesophyll cells of *Poa* are strongly labelled but with the highest density of silver grains found over haustoria (Figs. 8.20 and 8.22). Radioactivity also occurs in the host cytoplasm, particularly between the haustorium and the host nucleus (Fig. 8.21a,b), and immediately around the host nuclei as well as in the intercellular hyphae (Figs. 8.20 to 8.22). Sections cut longitudinally through the epidermis at the margin of a developing urediosorus, in a plane parallel to the leaf surface, occasionally show narrow zones of heavily-labelled fungal cells, penetrating between adjacent epidermal cells, in some of which the host nucleus is covered with a dense deposit of silver grains (Figs. 8.23 and 8.24). The concentration of label suggests that such emerging uredia are an important sink for [^{3}H]-glycerol. Sections in a similar plane, passing through more mature regions of uredia (Figs. 8.25 and 8.26) show a high concentration of radioactivity in urediospores and to lesser extent in closely associated structures. At this stage of development, relatively little label is detected in the mature uredial peridium (Fig.8.26a,b) and

paraphyses (Fig. 8.26a-d), which may already have been formed before the time of incorporation of label.

8.2 Quantitative comparison of the distribution of label from ^{3}H-glycerol in uninfected and infected leaf tissue

8.2.1 *Tussilago*

Photometer measurements of incident light reflected from silver grains lying over different areas of tissue in autoradiographs of *Tussilago* leaf sections are shown in Table 8.1. Since the large mesophyll cells of *Tussilago* frequently exceed the size of the 50 µm reflectance field, on which the data are based, separate mean values are recorded for the central vacuolar region and for the cell margin, with its extremely thin lining of cytoplasm, cell wall and, where present, intercellular hyphae, traversing the field of measurement as a narrow band. In the vascular tissue, however, due to the much smaller diameter of the cells, reflectance fields include a substantial proportion of the components of individual vascular strands.

Uninfected mesophyll cells are consistently more heavily labelled than cells of infected tissue, radioactivity being associated particularly with the chloroplasts and the peripheral cytoplasm, and only a low level present in the vacuolar region. Cell margins from the infected mesophyll, on the other hand, show only a low uptake of label from glycerol, similar to that retained by the vacuolar regions of uninfected tissue. The vacuoles of infected cells consistently give reflectance values below the background level. Vascular tissue passing through uninfected regions of *Tussilago* leaves is also more heavily labelled than that of leaf veins of infected tissue.

The relatively high density of silver grains over the sub-hymenial stroma, hymenium and aeciospores, already seen in photomicrographs of autoradiographs (Figs. 8.13 to 8.17), is borne out by reflectance measurements from these regions, which indicate that the aecium represents a sink for the labelled substrate supplied to the host. Within the aecium, the greatest concentration of radioactivity is found in the aeciospores and somewhat lower levels in the hymenium.

8.2.2 *Poa*

The results of quantitative assessment of the distribution of radioactivity in uninfected and infected tissues of *P. pratensis*, after feeding with ^{3}H-glycerol, are summarised in Table 8.2. The highest level of labelling of mesophyll cells occurs in infected tissue, corresponding particularly with the high density of silver grains, already noted over cells which contain haustoria and the somewhat lower level of radioactivity associated with intercellular hyphae (Figs. 8.20-8.22). In contrast to the uptake of label from ^{3}H-glycerol by *Tussilago*, infected mesophyll tissue of *Poa* accumulates more tritium than uninfected cells, either within the infected region ($P>0.001$) or at some distance from the infection ($P>0.01$).

Unlike the situation in *Tussilago*, vascular tissue in both infected and uninfected regions of *Poa* leaves is more radioactive than mesophyll tissue in its vicinity, whether infected or uninfected. Vascular strands passing through infected areas of *Poa* leaves are significantly more radioactive than those passing through uninfected tissue.

The relationship between infected and uninfected mesophyll tissue of *Poa* and the corresponding veins is also illustrated by the ratios of their reflectance values, summarised in Table 8.3. Within the infected region, infected cells clearly take up a much higher proportion of the available label from veins than do the small areas of uninfected tissue within this region. On the other hand, the ratios of radioactivity in mesophyll cells of infected and uninfected regions to that in the corresponding veins do not differ significantly.

Table 8.1 Effects of infection with *P. poarum* on the distribution of label from ^{3}H-glycerol in leaf tissue of *T. farfara*.

	Mean reflectance measurements[a]	
	Uninfected region[b]	Infected region[c]
Cell margin	26.82 ± 2.302	3.42 ± 0.511
Cell centre	3.07 ± 0.449	-0.44 ± 0.41
Vascular bundle	92.02 ± 15.811[d]	17.16 ± 1.898
Infected tissue[e]		3.36 ± 0.440
Sub-aecial stroma		12.27 ± 1.359
Hymenium		17.94 ± 1.726
Aeciospores		43.33 ± 13.489

a - Mean photometer response from five fields, 50 µm diameter, in each of five sections per slide ± standard error.

b - Mean values from 18 slides from four blocks of tissue.

c - Mean values from 5 slides from a single block of tissue.

d - Mean values based on 9 slides from 2 blocks of tissue.

e - Reflectance field including half of host cell (cell wall, peripheral cytoplasm, vacuole) plus intercellular hyphae.

Table 8.2 Effects of infection with *P. poarum* on the distribution of label from ^{3}H-glycerol in leaf tissue of *P. pratensis*.

		Mean reflectance measurements[a]	
		Uninfected region	Infected region
Mesophyll cells	Uninfected	40.01 ± 4.802	53.82 ± 7.048
		**	***
	Infected	-	78.78 ± 9.764
		***	*
Vascular strand		62.2 ± 9.589	88.66 ± 9.330

a - Mean photometer response from five fields, 50 µm diameter in each of five sections per slide ± standard error, based on 21 slides from five blocks of tissue.

Results of t-tests:

*** = differences exceeding 0.001 level of probability

** = differences >0.01 <0.001 level

* = differences >0.05 level

Table 8.3 Ratios of reflectance values of mesophyll tissues to veins in uninfected and infected regions of *P. pratensis* leaves.

	Uninfected region	Infected region
Uninfected cells	0.71 ± 0.103	0.55 ± 0.48 ***
Infected cells	-	0.86 ± 0.072

*** = differences >0.001 level of probability

CHAPTER 9

DISCUSSION

9

DISCUSSION

9.1 Spore formation and morphology of sori

This study has investigated in more detail aspects of the morphology and ontogeny of spores and sori of *Puccinia poarum* that had not been fully clarified, despite several studies in this respect having been made by Grove (1913), Wilson & Henderson (1966), Greene & Cummins (1967) and Henderson *et al.* (1972).

On account of their diagnostic importance, pycnia of rust fungi have been extensively studied (Craigie 1927; Hunter 1927, 1936; Cummins 1959; Hiratsuka & Cummins 1963; Gold *et al.* 1979; Gold & Littlefield 1979). Hiratsuka & Cummins (1963) described eleven types of pycnial morphology and other properties such as the growth pattern, bounding structures and the location of pycnia in the host tissue. According to this description, the rust *Puccinia poarum* has Type 4 pycnia, the characteristics of which are: (i) subepidermal pycnia; (ii) determinate growth; (iii) convex hymenia; (iv) well developed periphyses; (v) high frequency of occurrence on the Compositae. No diagnostic value was reported for flexuous hyphae by Hiratsuka & Cummins (1963). However, Burges (1934), as reviewed by Payak (1956), found flexuous hyphae in 53 rust species, distributed in 8 genera of Melampsoraceae and 8 genera of Pucciniaceae. The fusion of flexuous hyphae with pycniospores and its role in the

dikaryotization process have been previously reported (Craigie 1927, 1933; Buller 1950, Payak 1956). Although artificial transfer of pycniospores among pycnia is routinely used in our laboratory to induce the formation of aecia of *P. poarum*, no fusion of pycniospores with flexuous hyphae has been observed in this study nor have dikaryotic cells been found in the mycelium growing in the host tissues of aecial pustules. This latter point suggests that dikaryotization involves nuclear migration (Payak 1956; Craigie 1959, 1962), rather than the growth of dikaryotic hyphae from pycnia to protoaecia, unless the dikaryotic hyphae are sufficiently infrequent not to have been observed.

The transformation of periphyses into flexuous hyphae, which was reported by Buller (1950) in pycnia of *Puccinia graminis* at the light microscope level, has been confirmed in pycnia of *P.poarum* using both light and scanning electron microscopes. In *Scopella gentilis*, Payak (1956) reported that the flexuous hyphae formed from modified pycniosporophores. Such development has not been observed in the present work but the pseudoparenchymatous tissue surrounding the pycnium appears to be actively involved in the development of both flexuous hyphae and periphyses. The properties of the flexuous hyphae were summarized by Buller (1950) who reported that they were ephemeral in old pycnia of *Puccinia*. In this study, however, these structures have been observed in both young and old pycnia of *P. poarum*. Moreover, their number and lengths appear to increase with the age of the pycnium. Both hand sections and scanning electron microscopy are most useful techniques

for studying the occurrence and morphology of flexuous hyphae since these structures are usually destroyed in microtome sections. Penetration of the host epidermis similar to that shown by pycnia of *P. poarum* has been reported in other rust studies (Buller 1950; Hiratsuka & Cummins 1963; Gold *et al.* 1979). In general, the formation of pycniospores in *P. poarum* resembles that described by Littlefield & Heath (1979).

The present observations on the development of aeciospores of *P. poarum* and the ornamentation over their surface confirm those of Henderson *et al.* (1972) on the same rust. In the present study, however, both light and scanning electron microscopy show the presence of germ pores in aeciospores of *P. poarum*, features which were not observed by Henderson *et al.* (1972), but which are readily detected in light microscope preparations stained with cotton blue. Henderson *et al.* (1972) did not publish light micrographs to demonstrate the ontogeny of aeciospores, although they described, in one electron micrograph, the intercalary cells and aeciospores. The thickening observed in the wall of the aeciosporophores was not reported by Henderson *et al.* (1972). Similar remnants have been found in pycniosporophores and aeciosporophores of *Puccinia sorghi* (Rijkenberg & Truter 1974a,b). The presence of the annulations led Rijkenberg & Truter (1974b) to conclude that the development of aeciospore initials was annellidic rather than arthrosporic as described by Hughes (1970).

Henderson *et al.* (1972) studied the development of the ornamentations and refractive granules in aeciospores of *P. poarum* and demonstrated the taxonomic significance of the morphology of these

structures (Holm 1967, reviewed by Henderson *et al.* 1972) using a transmission electron microscope. The evidence of the present study suggests that the refractive granules may have formed over germ pores, which would support Henderson's interpretation of these structures as pore plugs. It has been suggested that the refracted granules may function as spore separators (Dodge 1924; Henderson *et al.* 1972). It appears equally possible, however, that they may serve in the attachment of aeciospores or germ tubes to the host. Figs. 3.29 and 3.30 show similar structures which appear to be involved in such attachment.

Several authors have mentioned the difficulty of separating uredial and telial stages of *P. poarum*, *P. poaenemoralis* and *P. recondita* (Wilson & Henderson 1966; Greene & Cummins 1967; Buchwald 1972; Preece, personal communication) owing to the conflicting observations which have been recorded. Klebahn (1914) and Gäumann (1959) as reviewed by Buchwald (1972) mentioned that no paraphyses were observed in teliosori of *P. poarum*. Wilson & Henderson (1966) indicated the presence of telial paraphyses and the absence of uredial paraphyses in *P. poarum* while variable development of paraphyses in teliosori and the usual absence of uredial paraphyses of this rust were reported by Greene & Cummins (1967), 'uredia mostly adaxial ..., usually without paraphyses but occasional specimens have short inconspicuous capitate, thin walled paraphyses'. However, on contrast to the above studies, the present investigation demonstrates that both uredial and telial paraphyses are frequently seen in uredia and telia of *P. poarum* respectively. Moreover, for the first time, the scanning electron miscoscope was

used to study the occurrence of the uredial and telial paraphyses of this fungus, allowing them to be distinguished easily from the spores.

Paraphyses as a term was first used in discomycetes (Boudier 1890) and is still generally used in rust fungi. Buchwald (1972) mentioned Petrak's recommendation of the term urophyses for the paraphyses of urediosori and proposed the term teliophyses to describe the sterile hyphal elements in telia of *P. poarum*. This study supports the use of these more precise terms to distinguish these characteristic sterile structures in teliosori in rusts from other fungi. The paraphyses of the uredial sorus may play some part in rupturing the epidermis and the release of urediospores. The appearance of the teliophyses in *P. poarum* suggests a protective function. Changes in their curvature on alternate drying and wetting (as used in our laboratory to induce teliospore germination, see McGee *et al.* 1973) might facilitate the rupturing of the epidermis and exposure of the teliospores.

Since the report by Cummins (1959), of the absence of peridia in uredia of *Puccinia* spp. and *Uromyces* spp., no further study of this aspect has been published. However, this investigation has revealed the presence of a peridium-like layer lying beneath the host epidermis. This layer, which is most readily seen in surface view of urediosori, was observed in both closed and opened uredia. Fragments of epidermis from macerated infected tissue show a regular network formation of brown fungal cell wall material adhering to the inner wall of the epidermis. A similar peridium consisting of a single layer of cells was reported in uredia of *Melampsora lini* (Hassan & Littlefield 1979).

As in other studies of rust fungi (Walker 1969), some teliospores are frequently present in mature uredia of *P. poarum*. Most teliospores are formed, however, in special teliosori of the type described here (p.45) and remain enclosed by the host epidermis for a prolonged period until suitable conditions for germination occur. The dormancy of teliospores in the intact teliosorus may be important in the life cycle of this macrocyclic rust.

In general the development of the urediospores and teliospores in this rust is essentially similar to that described by Harder (1976, 1977 respectively) (see also Littlefield & Heath 1979). The ornamentation on the surface of urediospores of *P. poarum* resembles that reported in other *Puccinia* spp. (Amerson & van Dyke 1978) and *Uromyces* spp. (see Littlefield & Heath 1979) where the spines are situated in a circular depression, and surrounded by annuli. Based on Murril (1905), Littlefield & Heath (1979) reported a list of teliospore ornamentation types. They divided the smooth surfaced teliospores into two categories, those without ornamentation and those with few small projections. Teliospores of *P. poarum* resemble the former category at lower magnifications but a shallow rugulose ornamentation is detected by scanning EM at higher magnifications. The accumulation of lipid droplets in urediospores and teliospores of *P. poarum* is similar to that reported by Williams & Ledingham (1964) and Harder (1977) respectively and clearly constitutes a major portion of the nutrient reserves of the spores.

In the present study, the measurements made on the external features of the pycnial, aecial, uredial and telial stages of this rust are generally in agreement with those reported by Wilson & Henderson (1966) and Greene & Cummins (1967).

Although monokaryotic pycnial-aecial infections result from exposure of *Tussilago farfara* plants to teliosori on *Poa pratensis* leaves, which have been subjected to drying and soaking (McGee 1973), light microscope and scanning electron microscope examination of such material has, so far, failed to reveal the expected metabasidia bearing basidiospores.

9.2 Intercellular and intracellular structures of *Puccinia poarum*

During infection with the macrocyclic rust, *Puccinia poarum*, cells of the pycnial-aecial host, *Tussilago farfara* are penetrated by apparently unmodified intracellular hyphae of the monokaryon, while typical rust haustoria of the dikaryon enter the cells of the uredial-telial host, *Poa pratensis*. Basically, these observations agree with the previous light (Rice 1927; Lösel & Lewis 1974) and electron (Rijkenberg & Truter 1973, 1974; Robb *et al.* 1975b; Harder 1978; Gold *et al.* 1979; Gold & Littlefield 1979; Glidewell & Mims 1979; Borland & Mims 1980) microscopic studies. The intracellular structures of the monokaryon, at the light microscope level, appear as relatively undifferentiated hyphae differing in frequency, growth habit, size and differentiation from the normal haustoria of the dikaryon in uredial and telial phases of rust fungi.

In *Tussilago farfara* infected with *P. poarum*, the intracellular structures have proved to be more frequent in mesophyll tissue than those reported by Lösel & Lewis (1974), although

much less abundant than on the uredial host. Using light microscopy, the present study found two slightly differing types of intracellular structures in pycnial-aecial pustules. Those recognized at the advancing margin of the colony are constricted at the point where they penetrate the host cell wall. The less specialized type, in the older parts of the pustule, where host tissue appears senescent, enters the mesophyll cells without constriction, suggesting a decreased resistance of host cells to fungal penetration at this stage. However, both types of intracellular hyphae appear as terminal, frequently septate, unbranched portions of the monokaryotic intercellular mycelium and, unlike the haustoria of the uredial and telial dikaryon, are not associated with a distinct haustorium-mother cell.

Sections of pycnial-aecial pustules parallel to the host epidermis allow an unusually clear view of the monokaryotic colony, radiating through the mesophyll tissue, and of its relationship with the pycnia and aecia. This has proved also to be a particularly favourable plane for observing the penetration of host cells by the fungus. In addition this study indicates the usefulness of light microscopic examination of resin embedded sections (0.5-1.0 µm thick) for studying the frequency of occurrence of septa in intracellular structures of rust fungi.

The increase in size and complexity of forms of haustoria throughout successive sub-stages of uredial and telial development of *P. poarum* on *Poa pratensis* does not appear to have been previously reported in other studies on rusts. Clearly there is a prolonged period of interaction with the host cell. In the

conditions under which the plants used in this work were grown, uredia and telia of *P. poarum* develop more or less in parallel, rather than in sequence. It is therefore unlikely that more advanced age of host tissue or of the rust infection is alone sufficient to account for the differences in morphology, pigmentation, density of intercellular fungal tissue and growth of haustoria observed between regions adjacent to telia and those adjacent to uredia.

The lower frequency of penetration of host cells by intracellular fungal structures in *Tussilago* and much denser inter cellular fungal tissue than in *Poa* suggest that in *P. poarum*, the intracellular hyphae of the monokaryon may be less important in nutrition than the haustoria of the dikaryon. Rijkenberg & Truter (1973) described the pycnial and aecial stages of various rusts as characterized by "a well-developed intercellular thallus and a relative dearth of intracellular structures" and concluded that the intercellular thallus may be "able to subsist largely on substances diffusing from host cells". They further commented: "The relatively unspecialized growth habit of pycnial and aecial mycelia may point to their potential culturability on axenic media, and may explain the wide host range of some pycnial and aecial rusts in contrast to the extreme host-specificity of uredial and telial stages".

Both light and scanning electron microscopy has confirmed the greater density of fungal growth in the intercellular space system of the pycnial-aecial host than of the uredial-telial host, noted in previous studies on *P. poarum* (Lösel & Lewis 1974; Lösel 1978)

and some other species (Colley 1918; Rice 1927; Gold & Littlefield 1979; Gold *et al.* 1979). The monokaryotic thallus is seen to grow densely and freely through the host mesophyll whereas the dikaryotic thallus is more limited in extent.

Light microscopy reveals that the ratio of haustorium volume to host cell volume is larger in bundle sheath cells of *Poa* than in mesophyll and epidermal cells. It is likely that this relates to physiological differences between these tissues and to their relative nutritional importance for the fungus, rather than to the actual volume of the cells.

Although differences in development of successive growth phases of *P. poarum* on its alternate hosts could result from differing physiological characteristics of the two hosts, as well as from alterations in pathological behaviour between monokaryon and dikaryon, there is much to suggest that the latter aspect is more significant. Pycnial and aecial growth stages of rusts frequently elicit greater disturbance of normal host physiology than the uredial and telial phases. In *P. poarum*, the monokaryon produces a relatively massive pustule region, much thicker and denser than the rest of the *Tussilago* leaf, largely due to the mass of fungal tissue present, rather than to hypertrophy or hyperplasy. From chitosan assays, Lösel & Lewis (1974) estimated that fungal tissue accounted for as much as 40% of the dry weight of *Tussilago* leaf tissue infected with pycnial-aecial stages of *P. poarum*.

The present study provides for the first time quantitative data on the distribution, frequency, size and development of intracellular structures on both alternate hosts of a macrocyclic

heteroecious rust fungus. The magnification and thickness of sections used in light microscopy are suitable for this type of assessment, since they allow observations on whole cells with their full complement of fungal intracellular structures.

In general, the intercellular hyphae of monokaryotic and dikaryotic phases of *P. poarum* are similar in structure. The significance of the absence of nucleoli in intercellular hyphae of the monokaryon and their presence in those of the dikaryon is uncertain but appears to correspond with observations of some other authors (Mendgen 1979; Glidewell & Mims 1979). The differences in septal structure between intercellular hyphae, with the normal perforate septum, and the pseudoparenchyma of uredia and aecia, with their partial or complete septa, agree with observations on other rusts reviewed by Littlefield & Heath (1979) and are of interest in relation to the functioning of these different regions of the fungal thallus. The present study appears to be the first to describe septa in both monokaryotic and dikaryotic hyphae of a single rust. Bilateral partial septa seen in dikaryotic hyphae of *P. poarum* have not been reported in other studies reviewed by Littlefield & Heath (1979).

Apart from the slight constriction where they cross the host wall, the intracellular hyphae of *P. poarum* resemble the intercellular hyphae in form, width and septation. In all the haustoria of the dikaryotic thallus of *P. poarum* so far examined by light or electron microscopy, only one nucleus has been observed in the haustorium body. A similar impression has been gained from electron micrographs of Mims & Glidewell (1978), Zimmer (1970), Robb *et al.* (1975b), Walles (1974) and Hardwick *et al.* (1971), although haustoria have also been described with two nuclei (Coffey *et al.* 1972a) or with

one nucleus in the body and another (as in Fig. 5.27 here) apparently migrating through the neck (Mendgen 1975). It is generally assumed that at maturity two nuclei are present in the haustorial body (Littlefield & Heath 1979) at which stage the haustorium-mother cell appears vacuolated and devoid of nuclei. It would be of interest to determine whether the nuclear arrangements within the haustorium correspond to different phases in nutrition and functioning of the structure. This study reveals that intercellular and intracellular structures of the monokaryon and those of the dikaryon differ in their amounts of nuclear materials as indicated by the higher proportion of heterochromatin to euchromatin in the dikaryon. No similar comparisons of nuclei of rust fungi during both dikaryotic and monokaryotic phases appear to have been reported.

It substantiated in further investigation, the ultrastructural evidence of glycogen in the monokaryotic thallus of *P. poarum* but not in the dikaryotic stages would be in agreement with its biochemical detection in leaf tissue of *T. farfara* infected with *P. poarum* (Holligan *et al.* 1974) and would correspond with the findings of Walles (1974) and Harder (1978). Other workers have not, however, found such a correlation (see review by Littlefield & Heath 1979).

In the present study, particular attention has been paid to differences in host wall reaction to penetration by intracellular hyphae and haustoria and to the extrahaustorial regions of these intracellular structures. The very narrow neck of the haustorium passing through the otherwise unchanged host cell wall suggests enzymic action at the penetration point unless, as found by Mims & Glidewell (1978) in *Gymnosporangium juniperi-virginianae*, the haustorium has entered via the plasmadesmata, which is not indicated by electron micrographs of this study. The abrupt interruption of the fungal wall, where the haustorium neck crosses the host cell wall, indicates some interference with fungal wall deposition at

this point. In contrast to this, the ingrowth of host wall material around intracellular hyphae of the monokaryon points to accelerated synthesis of host cell wall components and the continuity of uniform wall thickness from subtending intercellular hypha to intracellular hyphae excludes any interference with fungal wall synthesis.

Most sections of intracellular hyphae of *P. poarum* show deposition directly on the fungal wall, of variable amounts of material of moderate electron density (Figs. 5.36, 6.19a). The close correspondence in the appearance of this layer, the collar of material continuous with the host wall at the base of an intracellular hypha (Fig. 5.34) and the wall material which encloses a penetration peg (Fig. 5.26a) or encases hyphae growing parietally in host cells (Figs. 5.2, 5.41) suggests that it consists of wall material derived from the host. This view is consistent with the results of high resolution autoradiographic studies on other rusts (Mendgen 1975, 1979). The electron lucent region between the intracellular hypha and the host plasmalemma contains fibrils of similar electron density which appear to be contributing to the layer of host wall-like material. In contrast, the extrahaustorial matrix surrounding haustoria of this rust contains electron-dense fibrils, similar in density to the fungal wall, which seems to be adding to the fungal wall layer. No host wall-like material has been seen in the matrix around haustoria (extrahaustorial matrix). It thus appears that quite different processes are occurring around the haustorium and the intracellular hypha and that these may relate to differences in their functioning and interaction with host cells. However, in *P. poarum*, the region separating intracellular hypha and host plasmalemma is either electron-lucent or contains host wall-like material. Its composition is discussed in Section 9.4.5.

In their comprehensive review of the intracellular structures produced by rust fungi, Littlefield & Heath (1979) have compared the specialized haustoria, termed by them D-haustoria, produced by the dikaryon of many rusts, with the apparently unspecialized structures of the M-haustoria (P-haustoria *sensu* Harder 1978), which are here simply referred to as intracellular hyphae, produced by the monokaryon. The present observations agree with the relatively few records so far available of individual species throughout their life cycle (Littlefield & Heath 1979) in indicating that the change from monokaryon to dikaryon is characterized by the production of specialized haustoria, differing from the simpler intracellular hyphae of the monokaryon in their limited growth, lack of septa and their characteristic form, with a very narrow neck region, bearing an electron-dense neckband in the wall. In *P. poarum* there is a greater frequency of penetration of cells of the uredial-telial host by such haustoria than of the pycnial-aecial host cells by intracellular hyphae. There is also a more constant association of the host cell nucleus and other organelles with the haustorium of the dikaryon than with the intracellular hypha of the monokaryon. On the other hand, host chloroplasts are frequently associated with the intracellular hyphae. The intracellular structures of monokaryon and dikaryon of *P. poarum* differ also in the presence of a matrix between the intracellular hypha and the invaginated host plasmalemma, of material resembling the host cell wall, deposited directly on the wall of the intracellular hyphae, and its absence in the corresponding region around haustoria, where fibrils of similar electron density to the fungal

wall are observed. In addition, penetration by intracellular hyphae involves apparent ingrowth of host wall layers, while entry of the haustorium is accompanied by little disturbance of the host wall. On the other hand, the haustorium mother cell wall, unlike that of the intracellular hypha, is thickened at its point of contact with the host cell. This study has also shown differences in the nuclear components of monokaryon and dikaryon.

From the evidence of other studies on haustoria and intracellular hyphae (Harder 1978; Gold *et al.* 1979, Littlefield & Heath 1979) and from observations of hyphal and haustorial penetrations of mesophyll cells in *Tussilago* and *Poa* respectively, it is likely that both the membrane bounding the extrahaustorial matrix of the haustorium and that bounding the comparable region of the intracellular hypha, originate from the invagination of the host plasmalemma. The aggregation of membranes within the membrane bounding the intracellular hypha may be analogous to that observed by Harder *et al.* (1978), while the vesicle-like structure with electron-dense granules around intracellular hypha of *P. poarum* appears similar to the glandlike appendage of the sheath of *P. carthami* (Zimmer 1970). The origin and function of these vesicle-like structures could not be determined in this study, but it is possible to suggest that they may have a role in the exchange of substances between host and parasite.

As far as could be determined from the present investigation, intracellular hyphae terminate within the host cell and do not normally grow through and re-emerge from the mesophyll cells, as is seen in the scanning electron micrographs by Gold *et al.* (1979) of pycnial pustules of *Puccinia recondita*.

It is difficult to determine whether the embedding of monokaryotic hyphae of *P. poarum* in host wall material, the growth of such wall material around hyphal structures, the greater production of an apparently adhesive layer between hyphae and cell walls of *Tussilago* and the absence of all these features in tissues of *Poa* infected with the dikaryon, reflect special properties of the monokaryon or of the interacting host wall.

9.3 Invasion of vascular bundles

Colley's study (1918) of the macrocylic heteroecious rust **Cronartium ribicola** (cited by Buller 1950) described the penetration of the vascular bundle of the host by hypha-like 'haustoria' of the perennial, systemic, monokaryotic mycelium. In other rust studies (Jackson & Parker 1958; Jewell *et al.* 1962), changes in the anatomy of host vascular tissue have been reported but the association of the rust thallus with the host vascular system has attracted little further attention. The comprehensive review by Littlefield & Heath (1979) does not mention the invasion of vascular bundles by rust fungi.

The obvious nutritional advantage to the rust fungus of the presence of biotrophic hyphae in the vascular bundles does not appear to have been previously commented on. The close proximity of rust hyphae to conducting elements of xylem and phloem and, in particular, the presence of intracellular hyphae in specialized transfer cells of the phloem parenchyma of *Tussilago*, is likely to ensure an adequate presence of organic and inorganic nutrients and water. Although little is so far known of the permeability of the

fungal and host structures concerned, it is possible that such infection of vascular bundles may make the monokaryotic rust thallus relatively independent of other intracellular penetration.

In the case of *P. poarum*, as in the other rust species in which vascular infection has been studied (see Introduction), only monokaryotic phases invade the host vascular tissue. Longitudinal sections of vascular bundles of *Poa pratensis* infected with *P.poarum* show long haustoria in the large bundle sheath cells but no penetration of the heavily thickened endodermis and the vascular tissue enclosed by it. These observations have been confirmed by scanning electron microscopy. It thus appears that direct access to the vascular system is not important in the nutrition of the dikaryon.

In *Tussilago farfara* leaves, infection of vascular strands by the monokaryon of *P. poarum* has been observed using both light and electron microscopy. The penetration of xylem parenchyma and phloem companion cells of a herbaceous host by a rust fungus has been demonstrated for the first time. Light microscopy appears to show intracellular hyphae in xylem vessels of *Tussilago* leaves but electron microscope observations have, so far, shown fungal hyphae only in close contact with these tracheary elements. It seems that fungal hyphae associated with the walls of xylem elements may produce intracellular structures entering these vessels, as reported by Zimmer (1965) for safflower rust.

The presence of fungal structures in bundle sheath cells, phloem parenchyma and among xylem vessels and phloem elements corresponds with the situation in vascular tissue of *Rhamuus cathartica* infected with *P. coronata*, which was described without further comment by

Harder (1978). The indication of breakdown of some primary walls of xylem vessels is remarkably similar to the evidence of Harder's electron micrograph (1978). However, in both *Tussilago* and *Poa*, the breakdown of the primary walls of xylem vessels is observed also in vascular bundles from healthy and infected parts of the leaves. Similar observations have been reported on the vascular tissues of a variety of healthy flowering plants from both monocotyledons and dicotyledons (Esau 1965; review by O'Brien 1974).

In the vascular bundles of *Tussilago*, the differing wall structures associated with intracellular hyphae seen in transverse section suggest that the hyphal wall increases in thickness and electron density with age and becomes surrounded by a less dense layer of material resembling the host wall in texture and staining properties (Fig. 6.19a). A similar structure was found by Robb *et al.* (1975b) around intracellular hyphae of *Cronartium ribicola* in *Pinus* tissue cultures and by Harder (1978), forming a collar around the neck-like region of "P-haustoria" in the pycnial stage of *Puccinia coronata avenae* on *Rhamnus cathartica*.

The close similarity demonstrated in Fig. 6.19a between the membrane-wall association at the interface of the intracellular hypha with the host cell and at the host cell wall adjacent to an intercellular hypha in the vascular bundle region of infected *Tussilago* suggests that the same processes of wall deposition take place at both points. This interpretation is supported by Figs.6.15 and 6.20, where a similarly textured outer layer around intercellular hyphae projecting into the host cell is continuous with the host cell wall. The fibrillar material seen in some cases

(Fig. 6.19b) between the surrounding membrane and the wall of the intracellular hypha may represent an early stage of deposition of host wall-like material. The generally lower electron density of walls of intracellular hyphae in phloem parenchyma and xylem parenchyma than in bundle sheath cells and those of intercellular hyphae may also be physiologically important.

It has occasionally been suggested that the apparently empty sheath region around haustoria may be an artefact of fixation and Browning & Gunning (1977) have demonstrated that, while such a clear zone was present around wall proliferations of conventionally fixed transfer cells, it was absent in freeze-substituted preparations. In the present study, the absence of such a clear area around wall proliferations of transfer cells indicates that the region surrounding hyphae within the same cells is not an artefact. Similarly, in the host-parasite combination involving the dikaryon of the rust *Melampsora lini* infecting leaves of flax, Littlefield & Bracker (1972) found that the sheath region appeared similar in both frozen-etched cells and in those which had been fixed, dehydrated, embedded and sectioned. They also reported that the sheath was not an empty zone. However, the occasional distension of the region referred to above, suggests that it is not entirely rigid.

The presence of transfer cells of both type A (modified companion cell) and type B (modified phloem parenchyma) (see review by Gunning & Pate 1974) has been previously recognized in *Tussilago* Both type A and type B transfer cells, as well as unmodified phloem parenchyma and companion cells, are penetrated by monokaryotic hyphae of *P. poarum*.

The development of transfer-type cells has been shown to be induced by infection with viruses (Tu & Hiruki 1971), nematodes (Jones & Northcote 1972; Gunning & Pate 1974) or parasitic flowering plants (Cuscuta) (Gunning & Pate 1969), and is assumed in such cases to be a pathological condition (Gunning & Pate 1974). In contrast, the present investigation indicates a lower frequency of transfer cells in the vascular bundles from infected parts of *Tussilago* leaves than in those of healthy parts and the wall ingrowths of infected transfer cells appear fewer and less elaborate than in those of healthy vascular tissue. These observations may be of special importance in the physiology of the host-parasite relationship during the monokaryotic phase of rust development, where the involvement of the transfer cells of healthy vascular bundles in the absorption and secretion of solutes was suggested previously (Gunning & Pate 1974; Cutter 1978).

The less developed and less elaborate wall ingrowth of transfer cells in infected vascular bundles than in uninfected tissue may correspond to the reduced transport across these cells, which results from diversion of host assimilate to the fungus. Instead of being transferred from phloem parenchyma and companion cells into sieve tubes, much of the photosynthate from the leaf may be taken up by the intracellular hyphae. In such infected cells, the material in the electron-lucent region around the intracellular hypha appears very similar to the region between the host plasmalemma and wall ingrowths of uninfected transfer cells. It is possible that this region surrounding the intracellular hypha may be in some respects physiologically similar to that surrounding

the wall proliferations. The host cell wall-like material deposited on the hyphal wall surface may be comparable to the deposition of wall ingrowths and may be a response to transport into the hypha. The intracellular hyphae observed in the phloem region appear healthy and metabolically active and are generally thin-walled. In contrast to this, where dikaryotic haustoria in other species have been reported to be ensheathed in wall-like material, these haustoria are frequently senescent or necrotic (Heath & Heath 1971).

The lomasome-like structures in the transfer cells of *Tussilago* may resemble the "bud vesicles", described by Browning & Gunning (1977), or the "multivescular bodies" reported by Pate *et al.* (1969). Although, in Pate *et al.*'s study, the multivesicular bodies appear closely associated with the wall ingrowths, it seems that the plane of sectioning may remove these bodies from their original position which may correspond to the lomasome-like structure observed here. However, vesicles between cell wall and plasmalemma in plant cells have been previously reported (Walker & Bisalputra 1967).

Although *P. poarum* infects the vascular tissue of both lamina and petiole of leaves of *Tussilago*, no spreading of infection by longitudinal passage of the parasite through the vein has been observed. Unlike the fungal wilt infections of vascular tissue (such as *Fusarium oxysporum* and *Verticillium*, Dickinson & Lucas 1977), invasion of *Tussilago* leaf veins by monokaryon hyphae of *P. poarum* does not appear to be accompanied by adverse reactions of the infected host cells. This important relationship of biotrophic infection by pycnial-aecial stages of certain rusts with the vascular system of the host has previously been overlooked and has physiological implications which have still to be explored.

9.4 Host responses to infection by *P. poarum*

9.4.1 Host nuclei

The significance of the enlargement of the nucleus of infected cells and its close contact with the haustorium, which have been observed here in *Poa* and in various previous studies of rust infections (Hilu 1965; Zimmer 1965; Shaw 1967; Sood & Sackston 1970) remains a matter for speculation and may be of findamental significance in the host-parasite interaction. Nuclei of infected *Tussilago* cells do not usually show this obvious association with intracellular hyphae and, in mesophyll cells of *Tussilago*, the host nuclei enlarge much less than those in infected cells of *Poa*. These observations support the view that intracellular hyphae of the monokaryon differ from the haustoria of the dikaryon in their interaction with host cells. It is difficult to explain the reaction of epidermal cells to infection, where nuclei of *Tussilago* increase in volume to a greater extent than those of *Poa*, although the epidermal nuclei of *Poa* become unusually elongated.

The enlarged host nucleus of infected cells of *Poa* is frequently bent around or deeply invaginated by the haustorium (Fig. 7.15, 7.16). In their review Littlefield & Heath (1979) suggest that 'close association of the haustorium and the host nucleus is a characteristic response to invasion by M- and D-haustoria'. From certain previous studies (Colley 1918; Gold *et al.* 1979) as well as unpublished observations of M. D. Coffey and M. C. Heath, they conclude

that indentation of the nucleus by a haustorial lobe seems particularly common in cells invaded by M-haustoria. Further quantitative observations on a wide range of rust species at the light microscope level, such as have been made with *P. poarum* (see Chapter 4) may be required to clarify this point. The interaction which may exist between nuclei of haustoria and host cells is poorly understood. The close proximity of the fungal nucleus in the haustorium to the host cell nucleus, occasionally observed in the present study (Fig. 5.40), may be important at certain stages of development.

In addition to the alterations in form and size of nuclei in infected mesophyll cells of *Poa*, the electron micrographs reveal that the amounts of nuclear materials (euchromatin and heterochromatin) are markedly changed. Similar changes occur in the fine structure of nuclei in infected mesophyll cells of *Tussilago* where there is no close association of intracellular hyphae and host nucleus. Ultrastructural changes in nuclei of rust-infected tissue have been reported previously (Manocha & Shaw 1966; Robb *et al.* 1975a). Manocha & Shaw (1966) considered the heterochromatin regions as repressed DNA while euchromatin regions were active in RNA synthesis (Littau *et al.* 1964, reviewed by Manocha & Shaw 1966). However, Manocha & Shaw (1966) reported that the increase in electron density of euchromatin (interchromatin) in nuclei of mesophyll cells of rust-infected wheat was consistent with the increase in RNA and protein found in rust-affected nuclei (Bhattacharya *et al.* 1965). They also indicated that the decrease in the aggregation of heterochromatin might be equivalent to the decrease in the histone content (Bhattacharya *et al.* 1965). In general, the

changes in the structure of host nuclei in rust-infected leaves of *Tussilago* and *Poa* resemble those in uninfected, detached wheat leaves senescing on water (Shaw & Manocha 1965a; Shaw *et al.* 1965). The haustorium-host interaction is generally assumed to be mainly concerned with nutritional requirements of the parasite but it may directly involve the nucleic acid metabolism of the host (Chakravorty & Shaw 1977a,b). Further investigations are needed to study the changes in nucleic acids of both alternate hosts of *P. poarum*.

9.4.2 Chloroplasts

The ultrastructural changes observed in the chloroplasts of *Tussilago* and *Poa* infected by *P. poarum* essentially resemble those reported in various plants infected by other rusts (Shaw & Manocha 1965b; Orcival 1968; Coffey *et al.* 1972b; Heath 1974; Robb *et al.* 1975a; Abu-Zinada *et al.* 1975; Mlodzianowski & Siwecki 1975; Mares 1979), viruses (Esau 1968; Matthews 1973; Tomlinson & Webb 1978) or bacteria (Lallyett 1977), and during the processes of ripening (Thompson 1966; Spurr & Harris 1968), senescence (Ikeda & Ueda 1964; Shaw & Manocha 1965a; Ljubesic 1968) or chemical treatments (Butler & Simon 1968; Fischer *et al.* 1973; Heath 1974).

In *Tussilago*, the chloroplast ultrastructure during early stages of pycnial infection was similar to that observed in aged healthy cells, although osmiophilic globules were either absent or very few in the aged healthy cells. Robb *et al.* (1975a) reported an increase in the number of osmiophilic globules in chloroplasts of *Pinus* during pycnial infection by *Cronartium ribicola*. A similar increase was

clearly seen in chloroplasts of *Tussilago* during early stages of pycnial-aecial infection. The present study supports the observations of Orcival (1968) concerning the state of the chloroplasts of *Tussilago* in the pustule centre during later stages of infection with *P. poarum*. In the tissue adjacent to the pycnial-aecial pustule, however, there was no evidence of prolamellar bodies similar to those observed by Orcival (1968) in the corresponding red-coloured zone in his leaves. The changes in chloroplasts of infected *Tussilago* described here show a close resemblance to that demonstrated for virus-infected plants of other species (Esau 1968; Matthews 1973; Tomlinson & Webb 1978).

The chloroplasts in *Poa* mesophyll cells appear less sensitive to infection by this rust than those in *Tussilago*, where the chloroplasts show a relatively normal membrane system with an increase in number and size of strach grains and osmiophilic globules. The chloroplasts from the older healthy leaves of *Poa* contained a well-developed membrane structure with several, large strach grains. Similarly, a relatively unaffected chloroplast membrane structure was reported for rust-infected *Zea mays* (Van Dyke & Hooker 1969) and for cabbage plants infected by white blister fungus (Coffey 1975).

The significant reduction in chloroplast numbers in both alternate hosts of *P. poarum* after infection is in agreement with the observations of Sood & Sackston (1970) and Mares (1979) on other plants infected by rusts. The disappearance of the chloroplasts in *Poa* mesophyll cells infected by this rust was observed by Lösel (1978). The suggestion that the loss in chloroplast numbers in infected *Tussilago* might be attributed to the degeneration of the

chloroplasts, which appear to be controlled by both host and parasite, is supported by the presence of the host chloroplasts in close association with the intracellular structures formed by pycnial-aecial infections and by the reduction in the number of crystal-containing microbodies which have been reported to be a site of autolytic enzymes (Fredrick *et al.* 1968; Armentrout & Wilson 1969; Zimmer 1970; Coffey *et al.*1972; Beevers 1979). To explain the loss in numbers of chloroplasts in infected *Poa*, it is assumed that chloroplast reproduction decreases after infection (Novikoff & Holtzman 1970; Park 1976) whereas they continue to multiply in maturing healthy cells.

The peripheral vesicles observed in chloroplasts of healthy and infected mesophyll cells of *Poa* appear similar to those reported by Toyama (1980) in chloroplasts of Morning Glory leaves exposed to ethylene.

The changes in green colour of the tissue in both hosts of *P. poarum* may be related to the accumulation of carotenoids in the chloroplasts (Ikeda & Ueda 1964). However, in both hosts of *P.poarum* the responses of the chloroplasts to early stages of pycnial and uredial infections are similar, while the chloroplast responses in the alternate hosts differ during later stages of development of this rust. This indicates differences in the physiology of host-parasite interaction during monokaryotic and dikaryotic phases of *P. poarum*.

9.4.3 Microbodies in host cells

Although *Tussilago* mesophyll cells are larger in diameter than those of *Poa* cells, the number of crystal-containing microbodies in infected and uninfected *Poa* mesophyll cells is more than that in *Tussilago* cells. The reduction in number of microbodies in both infected *Poa* and *Tussilago* mesophyll cells after infection appear to correspond to the observations of Armentrout & Wilson (1969) and Zimmer (1970), who mention the rapid disappearance of microbodies from host cells soon after penetration by haustoria. Association of chloroplast and microbodies was frequently seen in infected cells where one or more crystal-containing microbodies normally lay close to the chloroplast. Such an association has been demonstrated for grasses and other plants (Fredrick & Newcomb 1969, 1971). However, the frequent association of these bodies with the host chloroplasts in rust-infected tissues was reported by Coffey *et al.* (1972b), who suggested here that the proximity of microbodies to the host organelles may be physiologically important. Fabbri & Palandri (1970) mentioned that crystals were not commonly seen in microbodies of the senescing tissue and their occurrence diminished with age. This corresponds with the absence of crystalline inclusions from microbodies of aged healthy cells of *Tussilago*. Microbodies in both alternate hosts and parasite resemble one another in general morphology but differ in their association with cell organelles. Microbodies in both *Tussilago* cells and monokaryon of *P. poarum* show less association with other cell structures than those in *Poa*. However, the occurrence of fungal microbodies near septa in Uredinales

(Maxwell *et al.* 1977; Littlefield & Heath 1979) appear equivalent to the association of microbodies and cell wall in *Poa* and *Tussilago* cells. In general, the associations of microbodies (peroxisomes or glyoxysomes) with other cell organelles in both host and parasite are thought to be functionally important (review by Maxwell *et al.* 1977). In *P. poarum*, no microbodies are observed in either haustoria of the dikaryon or intracellular hyphae of the monokaryon although microbodies are present in the intercellular hyphae. The present observations on fungal microbodies resemble those reviewed by Maxwell *et al.* (1977) in biotrophic fungi.

Several recent studies have indicated that plant microbodies contain catalase (Fredrick & Newcomb 1971; Tolbert 1971; Beevers 1979), and some other studies report an increase in peroxidase in rust-infected tissue (Macko *et al.* 1968; Seevers & Daly 1970). As suggested by Coffey *et al.* (1972b), it seems that the frequency of microbodies and the presence or absence of their crystals in both alternate hosts of *P. poarum* may be correlated with the production of the above enzymes which may be affected by the presence of the parasite.

9.4.4 Other host cell organelles

In spite of their association with intracellular structures of *P. poarum*, host mitochondria appear less affected than other cell organelles in the alternate hosts of this rust during early stages of infection. Changes in the structure of host mitochondria only occur during later stages of rust development in infected cells of

Tussilago, where these organelles appear either vesiculated or degenerated. It seems that the vesiculation of host mitochondria may represent a stage of degeneration of these structures. However, vesiculate cristae and crystalline inclusions similar to those reported by Coffey *et al.*(1972b) were not observed in mitochondria of either *Poa* or *Tussilago* cells after infection.

In infected mesophyll cells of *Poa*, Golgi bodies and rough endoplasmic reticulum are more numerous than in uninfected cells. The endoplasmic reticulum associated with haustoria of the dikaryon is ribosome-free. However, Golgi bodies and rough endoplasmic reticulum are not observed in infected and uninfected mesophyll cells of *Tussilago*. In response to rust invasion, an increase in the number of Golgi bodies and in the amount of endoplasmic reticulum in the infected cells have been previously reported (Van Dyke & Hooker 1969; Ehrlich & Ehrlich 1971; Harder *et al.* 1978; Littlefield & Heath 1979). The association of smooth endoplasmic reticulum and intracellular structures of *P. poarum* is consistent with other previous observations (see Harder *et al.* 1978). Harder *et al.* (1978) have suggested that the endoplasmic reticulum of the host may be involved in the synthesis of new plasmalemma-like membrane. However, additional studies concerning the function of the association of host cytoplasmic membranes and intracellular structures of rust fungi are required.

9.4.5 Host cell wall

An additional feature of infected mesophyll cells in *Tussilago* which is not found in *Poa*, is the presence of infoldings of host plasmalemma enclosing vesicles which, like the neck region of these

invaginations, contain granular material, similar in electron density to the host cell wall. These vesicles closely resemble those in parenchyma cells of *Helianthus* (Walker & Bisalputra 1967) and in hyphae of *Puccinia graminis tritici* (Thomas & Isaac 1967). Thomas & Isaac (1967) suggested that these bodies resemble lomasomes. Walker & Bisalputra (1967) reported that these vesicles originated in the cytoplasm, moved to the plasmalemma, fused with it, and emptied their contents on the cell wall. They indicated that the role of formation of these structures is similar to that described by Moore & McAlear (1961), and mentioned that the production and deposition of wall material may involve these types of vesicles.

In a number of studies it has been suggested that electron-lucent material associated with intracellular structures may be callose, a (1-3)-β-D-glucan found in characteristic locations in certain plant tissues, especially sieve areas of phloem (Currier 1957; Cutter 1978). Identification of "callose" has been largely histochemical and its structure and function are poorly understood.

Recently, Meier *et al.* (1981) have demonstrated that callose may be an intermediate in cellulose biosynthesis at the stage of secondary wall formation. Crafts & Currier (1963) (review by Cutter 1978) suggested that enzymes located in the plasmalemma are involved in the synthesis and degradation of callose; Cronshaw (cited by Robards 1974) assumed that callose degradation involves enzymes in the cell wall.

Currier (1957) reported several possible functional roles for callose: a) involvement in sealing and plugging action; b) prevention of leakage of sieve tube sap (or water) into the cell wall; c) structural role as a cell wall constituent; d) a possible intermediate in the degeneration and synthesis of the cell wall.

In the present investigation, both light microscopic observations on tissue stained with resorcinol blue and the distribution of fluorescence, after staining with analine blue, indicate the presence of callose around the monokaryotic intracellular hyphae of *P. poarum* and, at its point of entry, in the *Tussilago* cell wall. Infected and uninfected areas of both hosts show callose in their vascular bundles, a particularly clear reaction being found in xylem and phloem regions and in walls of both endodermal and epidermal cells. The fluorescence of the endodermal cell wall may indicate the presence of callose either as a wall constituent or related to cellulose deposition. However, no fungal structure of the dikaryon of *P. poarum* gives a positive reaction for callose while in *Tussilago*, callose is found around intracellular hyphae of the monokaryon and in the host cell walls adjacent to the point of penetration by the parasite. This appears to correspond to the deposition of host wall-like material around the penetrating hyphae suggested by the present electron microscopy and is supported by the observations of Meier *et al.* (1981) concerning the involvement of callose in the synthesis of cellulose. In certain resistant combinations of cowpea rust, Heath (1971) has described a callose sheath which grows up from the host cell wall around rust haustoria. The absence of callose around intercellular hyphae on both hosts suggests that the adhesive material between fungal and host walls differs in nature from that around intracellular hyphae.

9.4.6 Size of host cells

The only difference in host cell size in infected tissue compared with uninfected regions is the decreased size of mesophyll cells of *Tussilago* in the vicinity of pycnia. This effect, which is more readily observed in sections through mesophyll tissue, parallel to the epidermis, than in conventional transverse sections, suggests that, owing to competition with the rust thallus, these cells may have experienced less adequate nutrition during their development than cells remote from the infection. The absence of such an effect in *Poa* probably reflects the differing growth pattern of the grass leaf. Infection in *Poa* occurs in relatively mature regions of the leaf, which have completed their primary expansion closer to the basal meristem, whereas the dicotyledonous leaf probably continues growth and expansion of the lamina at and following the time of inoculation.

From light microcrope observations, it was found that the distortion and increased diameter of the infected parts of the petiole in *Tussilago* was due to the massive growth of the monokaryon, rather than to hypertrophy or hyperplasy.

9.5 Autoradiographic study of the distribution of label from ^{3}H-glycerol in infected and uninfected tissue

In both *Tussilago* and *Poa*, infection with *P. poarum* markedly alters the fate of ^{3}H-glycerol taken up by host leaves. On account of the technical difficulties encountered in a number of preliminary experiments in achieving an adequate level of labelling of tissue

for quantitative work, the present light microscope study suffers from the severe limitation of the quantitative assessments being confined to a single leaf of each host, although more specimens were available for qualitative observations. The overall agreement with the results of macroautoradiographic and biochemical investigations of similar tissues, using photosynthetically assimilated ^{14}C (Holligan *et al.* 1973; Fung 1975; Lösel & Lewis 1974; Lösel 1978) permits some confidence in the present observations.

As in previous studies with ^{14}C, radioactivity from ^{3}H-glycerol accumulates in infected areas of leaves of both hosts of *P. poarum* to a greater extent than in uninfected tissues. Analysis of microautoradiographs has made it possible to compare the effects of infection by monokaryotic and dikaryotic stages of *P. poarum* on the distribution of substrate, assimilated by the host, within tissues and within cells. Until now, there have been few attempts to apply microautoradiographic methods to studying rust infections at the greater level of resolution permitted by the use of tritium rather than ^{14}C (Bhattacharya & Shaw 1967; Favali & Marte 1973; Manocha 1975; Mendgen & Heitefuss 1975; Mendgen 1979) and no such study has been encountered in which both monokaryotic and dikaryotic stages of a single rust can be compared.

An indication of the profound physiological effects of infection on leaf tissue is seen in the chloroplasts of both hosts of *P. poarum*. Uninfected mesophyll tissue incorporates radioactivity from ^{3}H-glycerol mainly into chloroplasts, whereas infected tissue, particularly in *Tussilago*, shows much lower levels of label in chloroplasts. Microautoradiographic comparison of infected and uninfected tissues

shows a diversion of labelled substrates to the fungal thallus, with spores and spore-bearing structures as the principal sink. The more drastic alteration in chloroplast activity in *Tussilago* corresponds with ultrastructural evidence (Chapter 7) of greater disturbance of cells and chloroplasts of this host than of *Poa*.

In spite of the less severe effects on chloroplasts of infection of *Poa* leaves by the dikaryon of *P. poarum*, fundamental alterations in host metabolism are indicated by the greatly increased labelling of nuclei in ^{3}H-glycerol-fed tissue of *Poa* mesophyll and epidermis, paralleling the changes in nuclear size and ultrastructure recorded in Chapter 7. The observation by Bhattacharyo & Shaw (1967) of increased incorporation of label from tritiated cytidine and uridine into RNA of wheat leaves following infection with *P. graminis* var. *tritici* may be noted in this connection.

Further evidence of the differences in the relationship of monokaryotic and dikaryotic stages of *P. poarum* with their respective hosts is provided by the much greater intensity of labelling associated with haustoria than with intracellular hyphae of the monokaryon, which are not more heavily labelled than the intercellular hyphae. If the present indications of silver grains in autoradiographs lying around rather than within intracellular structures of the monokaryon of *P. poarum* are substantiated at the higher resolution of electron microscopic autoradiographs, it might be concluded that this accumulation of substrate from the host cell corresponds to the deposits of host wall-like material seen in electron micrographs of intracellular hyphae in mesophyll and bundle

sheath cells. This deposition may represent reaction by an incompatible host cell. At this level of resolution, however, it is difficult to be certain that the silver grains seen here are not associated with hyphal wall synthesis.

The situation in *Tussilago* could be comparable to that observed by Manocha (1975) in his comparison of resistant and susceptible host-parasite combinations involving *P. graminis*, where transfer of label from mesophyll cells of leaves provided with ^{3}H-leucine was apparently prevented by the deposition of "sheath" material on the haustorium wall within the extra-haustoria matrix in resistant hosts.

On present evidence, the monokaryotic hyphae, which invade mesophyll and bundle sheath cells of *Tussilago*, do not appear to be as important in the uptake of host assimilate as the haustoria of the dikaryon, with their intensive accumulation of radioactivity from ^{3}H-glycerol. The autoradiographic observations do not exclude the possibility that intercellular hyphae of *P. poarum* might obtain nutrients directly from host cell surfaces within the mesophyll. Andrews (1975) demonstrated the uptake by *Bremia lactucae* of label from lettuce cotyledons supplied with ^{3}H-glucose, even before penetration of the host, although, after infection, uptake was primarily via haustoria. In the case of *P. poarum*, however, it is likely that the vascular system, particularly the phloem, which is extensively invaded by the monokaryon, is the direct source of label incorporated by the fungus in *Tussilago*.

As far as can be determined at the present level of resolution, the intracellular hyphae within the vascular tissues of *Tussilago*,

which EM studies have generally shown to lack any deposit of host wall-like material in the matrix region between the fungal wall and the invaginated host plasmalemma, appear to show some radioactivity within the fungal cells. If this is substantiated by high resolution autoradiography, it may be concluded that, in *Tussilago*, the monokaryon obtains host assimilate directly from the vascular system and diverts it towards the fungal thallus and sporulating structures.

In *Poa*, on the other hand, where no vascular infection has been encountered, the nutrient requirements of the rust dikaryon appear to be supplied via the haustoria in mesophyll cells, which may continue both to photosynthesise and to maintain their normal supply of materials from the adjacent vascular system. Infection provides an additional sink for host assimilate and increases the activity of these pathways, as evidenced by the enhanced labelling observed in the infected mesophyll of *Poa* and adjacent vascular tissue. The much lower level of radioactivity of *Tussilago* leaf veins in infected than in uninfected parts of leaves may be due to direct uptake of translocated materials from the vascular tissue by the monokaryon of *P. poarum*.

Mendgen's (1979) observation of increased uptake of radioactivity by haustoria of *P. graminis* with increasing age appears to be supported in the present study by the density silver grains lying over long, folded haustoria. As in *P. graminis*, intercellular hyphae of the dikaryon of *P. poarum* are less heavily labelled than adjacent haustoria. From this difference in radioactivity and the relatively high grain density over host cell structures, Mendgen (1979) concluded

that transfer of ^{3}H-lysine from the host cells occurred via the haustorium to intercellular hyphae. He pointed to the contrast between this and the very low concentration of label in intercellular hyphae associated with a dead haustorium.

Surprisingly, although label from glycerol might be expected to accumulate preferentially in lipid-containing structures, the intercellular and intracellular hyphae of the monokaryon of *P. poarum*, which contain more lipid drops than the corresponding structures of the dikaryon, have been less heavily labelled than the dikaryotic structures in the conditions of the experiments described here. Very active accumulation of tritium is found in the readily-recognizable oil drops in mesophyll cells of *Poa* in the vicinity of the rust infection. These differences presumably reflect the relative rates of synthesis occurring in these various lipid bodies during the ^{3}H-glycerol-feeding and chase periods.

The most remarkable feature of microautoradiographs of sections of infected regions of leaves of both hosts of *P. poarum* is the intense labelling of spores and accessory structures, particularly in *Tussilago* where the increasing density of silver grains from the sub-aecial stroma to hymenium and aeciospores is in striking contrast to the low level of radioactivity in the infected mesophyll and intercellular hyphae. If the accumulation of radioactivity between aeciospores in the lower parts of the aecium, noted in Chapter 8, can be attributed to the breakdown of intercalary cells from the aeciospore stage one might speculate on whether this is functionally analagous to materials derived from the tapetum of flowering plant anthers during the development of pollen grains.

Autoradiographs of aecia also give clear evidence of involvement of tritium from ^{3}H-glycerol in wall synthesis of aeciospores and the aecial peridium.

The active labelling of all fungal structures in young uredia and the differentiation of intensely labelled urediospores from relatively unlabelled accessory structures such as uredial paraphyses and peridium observed in older uredia may correspond to the relative rates of development of these structures. In the latter case, the peridium and paraphyses must have developed before the period of exposure to ^{3}H-glycerol, during which the developing urediospores become labelled.

Seen in the context of the ultrastructural investigation already discussed, and previous biochemical studies, this preliminary analysis of the uptake of tritium by host and fungal structures goes some way towards elucidating the physiology of the association of *P.poarum* with its two hosts and has indicated certain important differences in the relationships of the mono- and dikaryotic growth phases with the corresponding hosts. The way has been prepared for further investigation by high-resolution autoradiography at the electron microscope level, which should lead to a better understanding of the fundamental processes of transfer of substrates from host tissues to the thallus of *P. poarum*.

9.6 Conclusion

The combined evidence of structural, ultrastructural and autoradiographic investigation has revealed several important differences between the monokaryotic and dikaryotic growth phases of *Puccinia poarum*, both in the structure and activity of host-parasite interfaces and in the degree of reaction of tissues of the alternate hosts.

As might be expected from the probable access of the monokaryon to nutrients translocated in the host phloem, which is indicated by its extensive invasion of the vascular bundles of *Tussilago*, the intracellular hyphae, which penetrate mesophyll cells, appear to be less important in the uptake of materials from the host than the haustoria of the dikaryon in leaf tissue of *Poa*.

A fundamental difference between the relatively unmodified intracellular hyphae of the monokaryon in mesophyll cells of *Tussilago* and the structurally specialized haustoria of the dikaryon is seen in the deposition of apparently host-derived wall material directly on the hyphal wall of the former, within the matrix region bounded by the host plasmalemma. This feature, which resembles certain resistance reactions by incompatible hosts in other rust infections, is absent in the extrahaustorial matrix of *P. poarum* growing on *Poa*.

The severe effects of infection on mesophyll cells of *Tussilago* particularly on the structure and functioning of chloroplasts, contrasts with the much lower level of disturbance of *Poa* mesophyll cells, in which chloroplasts remain structurally intact and continue

to assimilate nutrients, even when they contain haustoria, actively absorbing materials from the host cell.

It may be concluded that the monokaryon of *P. poarum* shows a less specialized parasitism than the dikaryotic phase of growth. The intimate association of haustorium and host nucleus, which is a constant characteristic of infection in *Poa*, may play an important role in this relationship.

APPENDIX

1. Lactophenol cotton blue (Shipton & Brown 1962)

Phenol	10 g
Glycine	10 ml
Lactic acid	10 ml
Cotton blue	0.2 g
Distilled water	20 ml

2. Sudan IV

To stain lipids, a saturated and filtered solution of Sudan IV in lactophenol was used. Sudan IV in lactophenol cotton blue, similarly prepared, was also used to stain lipids in occasional slides.

3. Preparation of cacodylate buffer

a) Preparation of 0.4 M solution of Na cacodylate

Na cacodylate	21.4 g
Distilled water to make	250 ml

b) Preparation of 0.2 M Na cacodylate

0.4 M Na cacodylate	50 ml	
0.2 M HCl	8 ml	(approximately for pH 7.2)
Distilled water to make	100 ml	

The pH of this buffer was adjusted to pH 7.0 with HCl.

4. Preparation of buffered glutaraldehyde

0.2 M Na cacodylate buffer	50 ml
25% glutaraldehyde	10 ml
Distilled water	40 ml
Final aldehyde concentration (%)	2.5

5. Preparation of buffered osmium tetroxide

Stock solution (w/v)	10 ml
0.2 M Na cacodylate	10 ml
Final concentration (%)	1.0

6. Preparation of Epoxy resin (medium hard mixture, i.e. 1A;3B)

Epon (Epikote) 812	117.85 g	93.26 ml
BEMA (accelerator)	3.025 g	3.30 ml
MNA (hardener)	82.525 g	68.05 ml
DDSA (softener)	30.900 g	31.00 ml (added last)

MNA - methyl nadic anhydride; DDSA - dodecyl succinic anhydride; BDMA - benzyl dimethylamine

7. Lead citrate (Reynolds 1963)

$Pb(NO_3)_2$	1.22 g
$Na_3(C_6H_5O_7).2H_2O$	1.76 g
Distilled water	30 ml

The mixture is shaken for one minute and then allowed to stand for 30 minutes with occasional shaking. 8 ml of 1 N NaoH is added and the suspension diluted to 50 ml with distilled water.

8. Cleaning solution

Potassium bichromate	100 g
Distilled water	850 ml
H_2SO_4 (conc.)	100 ml (added slowly with stirring)

Slides are soaked overnight in the cleaning solution, then washed for several hours in running tapwater, followed by 2 changes of distilled water, for 30 minutes each, and dried in a dust-free atmosphere.

9. Resorcin blue (Cheadle *et al.* 1953)

   0.25 g lacmoid is dissolved in 100 ml 30% ethanol. A few milliliters of 1% $NaHCO_3$ solution are added to keep the tissues slightly alkaline, in order to preserve the blue colour of the lacmoid.

10. Fluorescence microscope attachments used in the detection of callose

    High intensity mercury vapour lamp
    Exciter filter BG3
    Absorption filter BG38

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