STUDIES ON THE EXPRESSION OF RESISTANCE TO STEM RUST OF WHEAT CAUSED BY *Puccinia graminis* f.sp. *tritici*

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ABSTRACT

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The endogenous cytokinin levels of healthy primary leaves and seeds of a stem-rust susceptible wheat cultivar Little Club were compared with those of Little Club containing the stem rust resistance gene *Sr25*. Use was made of paper, column and high performance liquid chromatography techniques to separate the endogenous cytokinins in the plant material, and the soybean callus bioassay was used to test for cytokinin-like activity of the chromatography fractions. Leaf material of the resistant Little Club *Sr25* had a higher level of total cytokinin activity than Little Club, whereas seed material of Little Club *Sr25* did not always have higher levels of cytokinins than Little Club. A number of cultivars would have to be tested before the usefulness of cytokinin levels as an indicator of resistance could be determined.

The development of urediospore-derived infection structures of *Puccinia graminis* f.sp. *tritici* in wheat, barley, sorghum and maize was examined by scanning electron microscopy (SEM). Infection on and in the four species followed a similar pattern up to, and including, primary infection hyphae formation. In wheat, barley and maize, when a primary infection hypha abutted onto a host epidermal cell, a septum was laid

down delimiting a primary haustorial mother cell (HMC); primary HMCs did not form in sorghum. Secondary infection hyphae arose on the substomatal vesicle side of the primary HMC septum; infection did not progress further in maize, but in wheat and barley secondary infection hyphae branched, and proliferated intercellularly forming the fungal thallus. Secondary HMCs were delimited when an intercellular hypha abutted onto host cells. In all four species atypical infection structures were also observed.

In an attempt to determine the timing and expression of stem rust resistance gene *Sr5*, infection structure development of *Puccinia graminis* f.sp. *tritici* race 2SA2 in a resistant line (I*Sr5*Ra) and a susceptible line (I*Sr8*Ra) was compared quantitatively using a fluorescence microscopy technique. The results indicated that there were no significant differences in numbers of specific infection structures observed in the two near-isogenic lines up to, and including, 48 hpi, by which time race 2SA2 had successfully formed secondary HMCs in both lines.

PREFACE

The experimental work described in Chapter 1 of this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of Professor J. Van Staden. Research for Chapters 2, 3 and 4 was conducted in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, under the supervision of Professor F.H.J. Rijkenberg.

Chapters 2 and 3 have been combined and published [Lennox C.L. & Rijkenberg F.H.J. (1989) Scanning electron microscopy of infection structure formation of *Puccinia graminis* f.sp. *tritici* in host and non-host cereal species. *Plant Pathology* **38**, 547-556].

DECLARATION

I hereby declare that the studies presented in this thesis represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

C.L. LENNOX

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CONTENTS

CYTOKININS IN PLANT PATHOGENESIS

Growth and metabolism of plants are dynamic yet finely controlled processes, and years of research have revealed that plant hormones such as the auxins, cytokinins, gibberellins, ethylene and abscisic acid play vital roles in the regulation of plant growth and metabolism. Symptoms such as gall formation, stunting, tumour formation and epinasty, immediately indicate that the normal growth of the plant has been disturbed, and growth hormones have been implicated in a number of plant-pathogen interactions. Not so obvious is the involvement of growth regulators in diseases in which the symptoms do not involve gross morphological changes of the host. The green island phenomenon, in which the areas around infection sites remain green following leaf chlorosis is an example. Daly & Knoche (1976), Dekhuijzen (1976), Fraser & Whenham (1982), Pegg (1976a, b), Schröder (1987), Sequeira (1963, 1973), Surico (1986) and Tandon (1987) have reviewed the literature on the involvment of growth regulators in fungal, bacterial and viral diseases.

In the present investigation the relationship between endogenous cytokinins and resistance expression was examined. It was therefore deemed necessary to review the literature on the cytokinins and their potential role in microbial plant pathogenesis.

The discovery of cytokinins was a direct consequence of tissue culture studies by Skoog & Tsui (1948) and Jablonski & Skoog (1954). These workers found that coconut milk, extracts of vascular tissue, or malt extract, induced cell division in pith explants of *Nicotiana tabacum* L. during culture. Later, a purine-like compound capable of stimulating cell division was isolated. It proved to be an artifact resulting from the breakdown, during autoclaving, of herring sperm deoxyribonucleic acid (DNA). The highly active compound was identified as 6-furfuryl-aminopurine and termed kinetin (Miller *et al.*, 1956). Although kinetin does not occur in plant tissues

(Skoog & Armstrong, 1970), compounds exhibiting similar activity have been detected in plants. The first naturally occurring compound capable of inducing cell division was isolated from the endosperm of immature *Zea mays* L. caryopses and identified as 6-(4-hydroxy-3-methyl-trans-z-butenyl-amino) purine, or commonly termed zeatin (Letham, 1963; Miller, 1965). Since the isolation of zeatin, many other naturally occurring cytokinins have been extracted from diverse higher plant genera, bacteria, fungi and algae, as well as some insects (Letham, 1978), and Kende (1971) stated that as such, the cytokinins can thus be regarded as being ubiquitous.

Skoog & Armstrong (1970) defined the structural requirements for high-order cytokinin activity as being an intact adenine moiety with an N^6 - substituent of moderate molecular length.

A gradual documentation of largely circumstantial evidence has indicated that the roots are the prime site of synthesis, and today it is generally accepted that this is the case (Letham, 1978; Van Staden & Davey, 1979). Biosynthesis of free cytokinins in plant cells appears to represent a minute secondary pathway of the ubiquitous compound adenine, and the experimental difficulties created by this situation have greatly hindered progress towards elucidation of the pathway (McGaw *et al.*, 1984). There exist two schools of thought as to the biosynthesis of free cytokinins, namely that they are produced by the breakdown of tRNA (Klemen & Klambt, 1974; Maas & Klambt, 1981 a, b), or they are synthesized *de novo* (Burrows, 1978 a, b; Chen, 1981; Nishinari & Syono, 1980 a, b; Stuchbury *et al.*, 1979). Dickinson (1985) has stated that whereas there is no direct evidence for cytokinin production by the breakdown of cytokinin-containing RNA, there are definite indications suggesting the existence of pathways for the production of free cytokinins independent of tRNA turnover.

Cytokinins have been detected in both xylem sap (Gordon *et al.*, 1974; Hewett & Wareing, 1974; Horgan *et al.*, 1973; Letham, 1974; Purse *et al.*, 1976) and in phloem sap (Hall & Baker, 1972; Hoad, 1973; Phillips & Cleland, 1972; Van

Staden, 1976; Vonk, 1974;), and Van Staden & Davey (1979) stated that this indicates that these hormones are probably transported through both living and non-living translocatory tissue. Zeatin and ribosylzeatin are the major translocational forms of cytokinins in both xylem and phloem sap (Gordon *et al.*, 1974; Hewett & Wareing, 1974; Letham, 1974; Phillips & Cleland, 1972).

With regard to the fate of cytokinins, at least three possible metabolic routes should be considered (Van Staden & Davey, 1979): (1) that cytokinins are metabolised during utilization by being attached or incorporated into other molecules; (2) that they are broken down by catabolic processes and thus destroyed; and (3) that they are converted to "inactive" storage forms which, under certain conditions, may be reversibly sequestrated to "active" forms. While N $^{6}(\Delta^{2}$-*iso*-pentenyladenine, zeatin, dihydrozeatin, benzyladenine and their 9-ribosyl (and in the case of zeatin and dihydrozeatin their 0-glucosyl) derivatives, are generally very active, cytokinin activity is markedly reduced in the 7- and 9-glucosyl and 9-alanyl conjugates (McGaw, 1987). Cytokinin glucosides may be storage and bound forms (Parker & Letham, 1973; Van Staden, 1976) and work with endogenous cytokinins (Henson & Wareing, 1976) and labelled zeatin (Davey & Van Staden, 1981) has established that zeatin-like derivatives are transported to the leaves via the transpiration stream where they are metabolised rapidly to glucosylated forms. Thus glucosylation could occur whenever cytokinins are no longer required for active growth, providing the plant with a potential reservior of free cytokinins (Van Staden & Davey, 1979). Horgan (1987) reviewed current knowledge of the possible genetic control of cytokinin levels in plants via endogenous cytokinin and auxin biosynthetic and metabolic genes.

It is generally accepted that cytokinins are involved in cell division (Fosket *et al.*, 1977; Miller, 1961), they retard senescence by maintaining chlorophyll content, photosynthesis and chloroplast structure (Dennis *et al.*, 1967), and by maintaining protein and nucleic acid synthesis (Osborne, 1962; Richmond & Lang, 1957) and that they bring about nutrient mobilization within plant tissues (Mothes *et al.*, 1959; Mothes & Engelbrecht, 1961; Mothes *et al.*, 1961). Patrick (1987) stated that the

potential role of endogenous hormones as regulants of assimilate transport awaits clarification.

The level or site at which cytokinins are active within the cell is not known. The mode of action of cytokinins is poorly understood and insufficient evidence exists to identify any biochemical point of action conclusively (Horgan, 1984). However, they appear to exert their effect on plant metabolism as mediators, promotors or inhibitors of growth at a level close to, although not necessarily at, the genome (Burrows, 1975).

A number of plant pathogens which induce gross morphological changes in their hosts have been shown to produce cytokinins in culture, and the infected host tissue often has elevated levels of cytokinins when compared to the levels in healthy tissue. It is for these reasons that the cytokinins have been implicated in the development of abnormal growth after infection.

Barthe & Bulard (1974) working with *Taphrina cerasi* (Fuckel) Sadebeck, the organism that causes witches'-broom on cherries (*Prunus cerasus* L.), identified zeatin in the culture media of the fungus, and Kern & Naef-Roth (1975) working with a number of *Taphrina* species, identified zeatin and *iso*-pentenyladenosine in the culture filtrates of all the species examined. Sziraki *et al.* (1975) found that the neoplastic tissue of peach leaves (*Persica vulgaris* Mill.) induced by *Taphrina deformans* (Berk.) Tul. had increased cytokinin and auxin levels and a new cytokinin, not present in healthy tissue, was detected. This seemed to indicate the active production of cytokinins by the fungus and not just an alteration in the normal metabolism of cytokinins.

A number of cytokinins were identified in the culture media of *Pseudomonas syringae* pv. *savastanoi* (Smith) Young, Dye & Wilkie, the causal organism of olive knot disease (Surico *et al.*, 1975). The investigation of Surico *et al.* (1985) showed that both indoleacetic acid and cytokinins are needed to form the knots, and the size and anatomy of the knots are controlled by the balance of the two

growth regulators. When assayed during mid-log growth phase, cultures of wild-type *P. syringae savastanoi* produced 1 000 times more cytokinin than comparable cultures of *Agrobacterium tumefasciens* (Smith & Townsend) Conn. (MacDonald *et al.*, 1986). Cytokinin biosynthesis in strains of *P. syringae savastanoi* is, in part, specified by plasmid-borne genes (MacDonald *et al.*, 1986; Roberto & Kosuge, 1987).

Corynebacterium fascians (Tilford) Dowson, which causes fasciation or leafy gall on many annual or perennial herbaceous ornamentals, has been found to produce cytokinins in culture (Helgeson & Leonard, 1966; Thimann & Sachs, 1966). This organism has been shown to stimulate lateral bud growth in host plants, an effect also attributed to cytokinin action (Whitney, 1976). Rathbone & Hall (1972) found that at pH 7, *C. fascians* releases small amounts of *iso*-pentenyladenosine, whereas under acid conditions, as used by many other researchers, highly elevated levels of *iso*-pentenyladenosine were recorded. The release of *iso*-pentenyladenosine from tRNA of the bacterial cells under acid conditions is thought to be responsible for these elevated levels. Thimann & Sachs (1966) suggested that the bacterium stimulates the host tissue production of cytokinin by modifying the host metabolism, or by supplying a precursor from which the cytokinin is readily formed.

Clubroot diseases of cruciferous plants is found wherever plants of the mustard family grow. This disease is caused by a member of the Plasmodiophorales, *Plasmodiophora brassicae* Woron., and symptoms consist of small or large spindlelike, spherical, knobbly, or club-shaped swellings on the roots and rootlets. Serious losses are incurred when susceptible varieties of any cruciferous species are grown in infested fields (Agrios, 1988). Explants of tumour tissue have been found to produce callus on tissue culture media which do not have added growth substances, such as auxins or cytokinins. However, the presence of active vegetative plasmodia in the cells is essential for growth of the callus (Dekhuijzen & Overeem, 1971; Ingram, 1969). Clubroot tissue has been found to be three times more active in a cytokinin bioassay than healthy root material and partially purified

extracts from healthy and clubbed roots co-chromatographed on paper and on thin layer silica gel with zeatin and zeatin riboside (Dekhuijzen & Overeem, 1971). Dekhuijzen (1981) showed that the contents of bound and free cytokinins are different in host cell cytoplasm and plasmodia of the pathogen, and proposed that the plasmodia release cytokinins into the host cells. Evidence for direct biosynthesis of *trans*-zeatin from adenine by young plasmodia was found by Müller & Hilgenberg (1986).

Crown gall of woody and herbaceous plants is worldwide in distribution and is characterised by the formation of tumours or galls at the crown of the plant. Wyndaele *et al.* (1985) found that tissue from the green tumour line of soybean crown gall had two to three times higher cytokinin levels when compared to tissue from the pale line. The causal organism of this disease, *Agrobacterium tumefasciens*, has been shown to contain a class of large plasmids, the Ti plasmids (Zaenen *et al.*, 1974). Upon infection, a portion (the T-DNA) is transferred to the host plant cells and is replicated there (Chilton *et al.*, 1977). Once present in transformed tissues, the T-DNA is transcribed to RNA (Drummond *et al.*, 1977) which is presumably then translated. It has been suggested that the T-DNA codes directly for cytokinin biosynthesis and may effect the endogenous cytokinin levels (Garfinkel *et al.*, 1981). Cytokinins have been found to accumulate in the culture media of tumour tissue (Palni, 1984), and a complex of several cytokinins has been found to be responsible for tumourigenesis in the crown gall of tomato (Nandi *et al.*, 1989).

Many micro-organisms have been shown to produce cytokinin-like substances in culture (Mahadevan, 1984), and production has been found to increase during the formation of fungal fructification organs (Vizárová, 1975a). Greene (1980) reviewed the literature on cytokinins produced by micro-organisms and stated that it is possible that microbes originally obtained the genetic information necessary for zeatin synthesis from plants. The *iso*-pentenyl group of cytokinins is the most common of these growth regulators detected in fungi (Johnstone & Trione, 1974).

Both pathogenic and non-pathogenic isolates of *Cylindriocarpon destructans* (Zinssm.) Scholten have been shown to produce cytokinin-like substances in culture (Strzelczyk & Kampert, 1983), but no correlation could be found between pathogenicity of fungal isolates of *Cylindrocarpon destructans* (Kriesel, 1987) or *Fusarium culmorum* (W.G.Sm.) Sacc. (Michniewicz *et al.*, 1984) and their ability to produce cytokinin-like substances. Surico (1986) and Surico *et al.* (1985) on the other hand, found that the bacterial strains of *Pseudomonas syringae* which were capable of producing high amounts of indoleacetic acid and cytokinins were more virulent pathogens. Virulence assays indicated that both indoleacetic acid and cytokinins function as virulence factors in this plant-pathogen interaction (Roberto & Kosuge, 1987).

Green island is the term used in reference to a ring or spot of living green tissue which is centred around an infection site and which is surrounded by yellowing (chlorotic) tissue. Both biotrophic and facultative microbial plant pathogens have been shown to produce green islands in nature (Bushnell, 1967), as have insect infections (Engelbrecht, 1971). Stakman (1914), as cited by Bushnell (1967), applied the term "green islands" to the spot of green that occurred with certain incompatible host-parasite combinations with stem rust (*Puccinia graminis* f.sp. *tritici* Eriks. & E. Henn.) of wheat (*Triticum aestivum* L.). This type of green island in part characterises infection type 2 with stem rust of wheat (Stakman *et al.*, 1962). Compatible combinations of host and parasite can produce green islands, but the islands are not usually seen unless the senescence of infected leaves is accelerated by a lack of adequate light, by darkness, or by detachment of leaves from plants. A leaf that is ageing slowly and normally in a well-lighted environment is less apt to show green islands than one that is yellowing rapidly in a suboptimal environment (Bushnell, 1967). On the other hand, Sziráki *et al.* (1976) noted that they regularly observed the appearance of green islands in both susceptible and resistance wheat-stem rust combinations.

The culture filtrates of a number of facultative plant-pathogenic fungi evoke the formation of green islands in detached host leaves (Suri & Mandahar, 1984, 1985;

Vizárová, 1975a; Yadav & Mandahar, 1981) and a mimicking of the green island effect has also been observed when water-soluble components obtained from the conidia of powdery mildew (*Erysiphe graminis* DC) (a biotroph) were applied to detached leaves of barley (*Hordeum vulgare* L.) (Bushnell & Allen, 1962; Vizárová, 1974a). Similarly, Angra & Mandahar (1985) found that green islands were produced in excised maize leaves (*Zea mays* L.) underneath spore suspension drops of *Drechslera carbonum* Ullstrup and *Bipolaris maydis* Nisikado after incubation in the dark.

Green islands were first described by Cornu in 1881 (Bushnell, 1967), and in spite of the many studies since, much controversy surrounds the formation of such islands (Scholes & Farrar, 1987). Cytokinins have been implicated in the formation of green islands as the exogenous application of these compounds to detached leaves has been shown to mimic green island formation induced by plant pathogens (Angra & Mandahar, 1985). These compounds have also been shown to delay senescence or effect a juvenile condition in plant tissues by delaying chlorophyll breakdown, enhancing protein synthesis and mobilizing metabolites, all of which have been shown to be characteristics of green-island tissue. However, exogenous application of cytokinin to intact plants does not result in green island formation (Atkin & Neilands, 1972) as the cytokinins are rapidly metabolized by the plant tissue (Fox, 1966).

Yadav (1981) found that green islands were produced on detached barley and wheat leaves by spore-containing infection drops of *Bipolaris sorokiniana* (Sacc.) Shoem. soon after host penetration had occurred. These green islands had higher cytokinin-like activity and a greater accumulation of sugars and starch than uninfected tissue (Yadav & Mandahar, 1981). The conclusion these researchers came to was that, as gramineaceous leaves do not produce cytokinins, the cytokinins were produced by the germinating conidia and by the fungal mycelium in the initial stages of pathogenesis, and that this creates translocatory sinks ensuring a regular supply of nutrients to the pathogen. Dekhuijzen & Staples (1968) found that none of the mobilization-promoting fractions in urediospores and isolated

bean rust, *Uromyces phaseoli* (Pers.) Wint., mycelium had R_f values similar to those in extracts from infected leaves of bean plants which had been placed under low light intensities to encourage green-island formation. They implied that factors leading to green island formation are strictly of host origin.

Another point of controversy in green island formation is whether there is a continuous maintenance of, or increase in, chlorophyll throughout disease development as proposed by Bushnell (1967), Scholes & Farrar (1987) and Sziraki *et al.*, (1984), or whether there is an initial breakdown of chlorophyll followed later by "re-greening" (Allen, 1924, 1926; Allen, 1942; Mares & Cousen, 1977; So & Thrower, 1976).

Aggab & Cooke (1981) reported observing that tissues surrounding sites of sclerotium formation of *Sclerotinia curreyana* (Berk.) Karst. in *Juncus effusus* L. culms remained green, while general chlorosis occurred in the culm tissue. They found that the highest chlorophyll levels occurred in the sclerotium-surrounding tissue, and stated that maintenance of the host's photosynthetic potential at sites of sclerotium differentiation ensures a supply of carbohydrate to the parasite during this critical stage of its development. Green-island tissue resulting from *Albugo candida* (Pers.) infection of *Brassica juncea* L. cotyledons was seen to have a five times higher $^{14}CO_2$ fixation than non-infected tissue (Harding *et al.*, 1968) and chloroplast breakdown was delayed in the infected green-island tissue. Camp & Whittingham (1975), working with powdery mildew infected barley leaves, found that although the chloroplasts of green-island tissue were enlarged and fewer in number than healthy tissue, they retained their green colour because of sufficient pigment synthesis and adequate chloroplast lamella number.

Whenham (1989) found that green islands, induced in tobacco (*Nicotiana tabacum* L.) leaves by systemic tobacco mosaic virus infection, contained a reduced concentration of free cytokinins and exhibited an increased rate of cytokinin catabolism. This author suggested that increased free cytokinin concentration is not involved in biogenesis of green islands.

Possibly the specific peculiarities of each of the plant-pathogen interactions have added to the confusion over the formation of green islands, and as such each interaction should be considered on its own.

From the observations that many facultative plant pathogens have been shown to produce cytokinin-like substances in culture, it is feasible to assume that these substances may contribute to the cytokinin pool of infected plants.

The fact that the infection of plants by fungal pathogens might alter the quality of cytokinins is presented and discussed in the paper by Mills & Van Staden (1978). Wheat plants infected by *Fusarium culmorum* were found to have more auxin-like, gibberellin-like and cytokinin-like substances than healthy plants, and a quality change in cytokinin was detected after infection (Michniewicz *et al.*, 1986a).

Bist & Ram (1986), investigated the malformation of mango inflorescences (*Mangifera indica* L.) and found that cytokinin changes in healthy and malformed tissues followed a similar pattern, although cytokinin concentrations were always higher in the malformed inflorescences. Some qualitative differences were detected between chromatographs of cytokinins from malformed and healthy inflorescences, and they concluded that these changes were probably due to the association of fungi reported to be present in malformed panicles. Most workers consider *Fusarium moniliforme* var. *subglutinans* Wr. & Reink. to be the responsible organism for malformation, although data provided are not absolutely conclusive (Nicholson, 1986). Higher levels of endogenous cytokinins in material from malformed mango inflorescences than that from healthy inflorescences were also reported by Nicholson & Van Staden (1988), as were qualitative differences in the cytokinin complement extracted from healthy and malformed inflorescence material. The presence of *iso*-pentenyladenine in malformed flowers (Nicholson & Van Staden, 1988) and in cultures of *Fusarium moniliforme* var. *subglutinans* (Van Staden & Nicholson, 1989), but not in healthy inflorescences, implies that the fungus is creating a hormonal imbalance in malformed inflorescences. Studies of *Fusarium moniliforme* var. *subglutinans* in culture have shown that this

fungus can synthesize cytokinins, notably *iso*-pentenyl adenine and *trans*-zeatin (Van Staden & Nicholson, 1989). Van Staden *et al.* (1989) stated that the extent to which the fungus can interconvert the synthesized compounds is relevant to the possible involvement of cytokinins in flower malformation. From the results of experiments using [^{3}H]*iso*-pentenyladenine and [8-^{14}C]*trans*-zeatin fed *Fusarium moniliforme* var. *subglutinans* cultures, these authors concluded that the major contribution of the fungus to cytokinin production in the mango flower may be that it rapidly produces *iso*-pentenyl derivatives and/or converts *trans*-zeatin to such derivatives, thus reducing the production of dihydrozeatin compounds necessary for normal growth, flower development and fruit production.

Both work on rust diseases (Dekhuijzen & Staples, 1968; Kiraly *et al.*, 1967; Sziraki *et al.*, 1976; Vizárová *et al.*, 1986) and powdery mildew diseases (Kern *et al.*, 1987; Mandahar & Garg, 1976; Vizárová, 1974a, b, 1975b, 1979, 1987) have shown an increase in cytokinin activity with infection.

There has been much debate as to the source of the cytokinin increase in infected plants. Yadav & Mandahar (1981) were of the opinion that these increased levels reflect secretion of cytokinins by the pathogen. Such cytokinins would create localized translocatory sinks towards which nutrients would move from the surrounding areas. Dekhuijzen (1976) however, concluded that infection stimulates the production of cytokinins by the host plant. Dekhuijzen & Staples (1968) found that although the urediospores and mycelium of bean rust have cytokinin-like compounds, these are not the same as those found in infected tissue. Thus they conclude that the cytokinin increase observed is strictly of host origin. Qualitative changes in cytokinins observed in barley and wheat cultivars after infection by powdery mildew (Vizárová, 1974b, 1979, 1987; Vizárová *et al.*, 1986) and stem rust (Vizárová, *et al.*, 1986; Vizárová, *et al.*, 1988) indicate that the products of the pathogen do have an influence on the cytokinin metabolism of the plant. At this stage, the extent to which the pathogen contributes to the cytokinin pool of the infected plant, or the level at which products of the pathogen interfere with the metabolism of cytokinins in the infected plant is unclear.

The manipulation of the host's metabolism by the pathogen is a key factor in the establishment of the complex interaction between obligate parasites such as the rust and mildew fungi (Barnes *et al.*, 1988). Many of the changes in metabolism of host plants detected after infection could be, at least in part, attributed to increased cytokinin activity in the infected tissue.

The mobilization of metabolites and the accumulation of substances in the infected tissue has been shown by a number of authors (Allen, 1942; Dekhuijzen & Staples, 1968; Hwang *et al.*, 1986; Kiraly *et al.*, 1967; Livne & Daly, 1966; Poszar & Kiraly, 1966; Shaw, 1961). This abnormal transport of nutrients to the locus of infection has been shown to be at the expense of young actively growing tissue, which as a result is ultimately smaller is size (Livne & Daly, 1966; Poszar & Kiraly, 1966). Ahmed *et al.* (1982) found potassium and phosphorus to accumulate in barley leaves infected with brown rust. This, they state, can be explained entirely by relatively unaltered xylem import into diseased leaves and reduced export of the phloem-mobile ions, and that there is no confirmation of production of cytokinin-like substances by the fungus which directs transport to infection areas.

Delayed senescence of infected tissue has been found to be due to delayed chloroplast break-down and chlorophyll retention (Mukherjee & Shaw, 1962; Singh *et al.*, 1982; Sziraki *et al.*, 1984). The higher levels of all photosynthetic pigments during later stages of pathogenesis can be explained by increased synthesis in the diseased leaves (Singh *et al.*, 1982). Elevated levels of nucleic acid have also been detected in infected tissues (Barnes *et al.*, 1988; Chakravorty *et al.*, 1974; Heitefuss, 1966; Manners & Scott, 1984) and these could contribute to delayed senescence. Such a delay in the onset of senescence could be a great ecological advantage to an obligately parasitic fungus in allowing its continued growth and sporulation (Harding *et al.*, 1968).

Detached wheat leaves, when floated on water, retain their green colour for a few days only and are usually chlorotic within a week. However, leaves floated on 30 - 100 p.p.m. benzimidazole retain their green colour and their capacity to support

growth of leaf and stem rust for periods of up to a month (Person *et al.*, 1957). With detachment, normally incompatible reactions of attached leaves are altered to greater susceptibility (Forsyth & Samborski, 1958; Mayama *et al.*, 1975). This breakdown of resistance can be prevented by floating leaves in solution of benzimidazole or kinetin (Cole & Fernandes, 1970; Dekker, 1963; Edwards, 1983; Person *et al.*, 1957; Samborski *et al.*, 1958; Shaw, 1963; Wang *et al.*, 1961). Contrary to these findings, Mayama *et al.* (1975) found that floating the leaf pieces on kinetin did not prevent the increase in susceptibility. Cole & Fernandes (1970) and Edwards (1983) reported an actual increase in resistance by treatment with cytokinin. Liu & Bushnell (1986) were of the opinion that in these cases, kinetin may have directly inhibited fungus development instead of enhancing host resistance, especially in view of the inhibitory effects of kinetin on development of the powdery mildew fungus in their own study, and in that of Edwards (1983).

Enhancement of the hypersensitive reaction (HR) by kinetin has been shown for stem rust of wheat (Mayama *et al.*, 1975), in which the HR sites were more numerous and larger than in attached leaves, and powdery mildew of barley (Liu & Bushnell, 1986), where there was a doubling in the number of cells that died at each infection site, suggesting that kinetin had increased the spread of killing factors beyond the cells that contained primary haustoria. Zeatin had no effect on the HR of barley to powdery mildew (Liu & Bushnell, 1986).

The development of a number of powdery mildew fungi was checked completely by floating inoculated host leaf disks on aqueous solutions of kinetin, but this compound was inactive against *Botrytis fabae* Sardina and *Uromyces appendiculatus* (Pers.) Unger (Dekker, 1963). Attempts to control powdery mildew (*Erysiphe cichoracearum* DC. ex Merat of intact cucumber (*Cucumis sativus* L.) plants, by application of kinetin solutions to bare roots and as foliar sprays, failed, and insufficient transport of the chemical in plant tissue could be a factor contributing to this failure (Dekker, 1963). Hopkins (1985) found that foliar applications of kinetin to grape cultivars (*Vitis vinifera* L.) susceptible to Pierce's disease (caused by a xylem-limited bacterium) did not prevent symptoms in

inoculated plants, whereas in a moderately resistant cultivar, kinetin prevented the development of symptoms and prevented the accumulation of the bacterium in the leaves, hence the cultivar became more resistant.

The effect of kinetin on the *in vitro* development of *Cylindrocarpon destructans* (Kriesel, 1987), and *Fusarium culmorum* (Michniewicz *et al.*, 1984) has been documented. At low concentrations (10^{-9} - 10^{-6} M), kinetin has no effect, or a slight stimulatory effect on spore germination (Kriesel, 1987; Michniewicz *et al.*, 1984), whereas at higher concentrations (10^{-3}M) germination is inhibited. Hyphal growth in culture was not affected at 10^{-9} - 10^{-6} M (Kriesel, 1987), is inhibited at 10^{-6} - 10^{-3}M kinetin (Kriesel, 1987; Michniewicz *et al.*, 1984) and stimulated at 10^{8} - 10^{6} M (Michniewicz *et al.*, 1984). Most sensitive to kinetin were fungi in the earlier phases of growth (Michniewicz *et al.*, 1984). Fungal sporulation was slightly stimulated by low concentrations (10^{-9} - 10^{-8}M) of kinetin, and was inhibited at higher concentration (10^{6} - 10^{-5}M) (Michniewicz *et al.*, 1984). No correlation was found between the pathogenicity of the isolates and their susceptibility to kinetin (Michniewicz *et al.*, 1984). Michniewicz *et al.* (1986b) found that the highest production level of cytokinin-like substances was present in five-day-old *Fusarium culmorum* cultures, that is, at a stage in which Michniewicz *et al.*, (1984) found the sensitivity of this fungus, to exogenous kinetin, to be low.

Kinetin treatment of detached leaves resulted in the formation of swollen appressoria of *Erysiphe cichoracearum* on tobacco (Cole & Fernandes, 1970), but had no effect on appressorium formation by *Erysiphe graminis* on attached leaves of barley (Liu & Bushnell, 1986). An inhibition of kinetin of appressorium formation of *Erysiphe graminis* on nitrocellulose membranes suggests that exogenously applied kinetin affects fungus development on the host directly rather than indirectly through changes in host cells (Liu & Bushnell, 1986). Haustorium development is inhibited by kinetin (Dekker, 1963; Liu & Bushnell, 1986) and the haustoria which do develop are usually malformed (Liu & Bushnell, 1986). Vizárová (1987) found zeatin and its derivative (at 100μg per 3 cm^3) to have an absolute inhibitory effect on the growth of *Erysiphe graminis* compared to kinetin and benzylaminopurine

which had only slight inhibitory effects. On the other hand, Liu & Bushnell (1986), could find no effects of zeatin (at concentrations of 10^{-6} - 10^{-4} M) on the development of this fungus on detached barley coleoptiles. It is possible that differences in time of application of the compounds, tissues used, and concentrations used, could account for these differences.

Barley cultivars resistant to powdery mildew have been shown to have higher levels of cytokinin activity before infection than susceptible cultivars and a close correlation has been found between resistance and cytokinin levels (Kern *et al.*, 1987; Vizárová, 1975b, 1979, 1987; Vizárová & Paulech, 1979; Vizárová, *et al.*, 1988). Higher levels of endogenous cytokinins were also found in dried seed of both barley and wheat cultivars resistant to powdery mildew, than in those of susceptible cultivars (Vizárová & Muzikova, 1981; Vizárová & Vozar, 1984; Vizárová, *et al.*, 1988), and Vizárová (1987) found that resistant cultivars of these two cereals have higher cytokinin activity in their entire ontogeny than susceptible cultivars. These results point to the possible important role of free endogenous cytokinins in the resistance of cereals against powdery mildew.

Vizárová and her co-workers have spent more than a decade investigating the rôle of endogenous cytokinins in the barley- and wheat-powdery mildew interaction (Vizárová, 1973, 1974a, 1975b, 1979, 1987; Vizárová & Kováčová, 1980; Vizárová & Minarcic, 1974; Vizárová & Muzikova, 1981; Vizárová & Paulech, 1979; Vizárová, *et al.*, 1988; Vizárová & Vozar, 1984). They have found that with infection of both resistant and susceptible cultivars, there is an initial decrease during fungal incubation (0 - 4 days post-inoculation, dpi), followed by a rapid increase until 6 dpi (when spore production is initiated in the susceptible cultivars). In the susceptible cultivar, the cytokinin activity continues to rise as spore production continues, whereas in the resistant cultivar a decline is noted at 6 dpi. In both resistant and susceptible cultivars, inoculated leaves had higher cytokinin activity than healthy leaves, however, the susceptible cultivars show a much greater overall increase than resistant cultivars. Similar changes in endogenous cytokinins of the 5th leaf of resistant and susceptible barley cultivars, inoculated with powdery

mildew, were noted by Kern *et al.* (1987).

Levels of endogenous cytokinins in root tissue of barley and wheat cultivars have been shown to change on infection of the above ground parts by powdery mildew fungi (Vizárová, 1973, 1974b, 1975b, 1979; Vizárová & Minarcic, 1974; Vizárová & Paulech, 1979; Vizárová *et al.*,1986). In root tissue of both resistant and susceptible cultivars, there is an initial increase in activity between 0 and 4 dpi, this increase being greatest in the resistant cultivar. In the susceptible cultivars, levels remained high until the first spores formed. Following this increase there was a steady drop back to a level near to that recorded in healthy root material of both the resistant and susceptible cultivars. The overall decrease in cytokinin activity of root material is greatest in the susceptible cultivars, indicating a greater removal from these roots.

Vizárová & Minarcic (1974) found that, associated with increased free cytokinin content of root material of a susceptible cultivar at 4 dpi, there was an inhibition of elongation growth, inhibition of growth and formation of lateral roots, and changes in morphology and anatomy of roots at segments related to 4 dpi. This, according to these authors, indicates a decreased translocation to above-ground parts on that day. They are of the opinion that at 4 dpi the parasite inhibits the transport of cytokinins from the roots to the leaves and in support of this opinion they cite Cole & Fernandes (1970) as having found that the cytokinins influence the growth of the parasite in a negative way. Thus the reaction of the parasite would be a defensive reaction.

Qualitative changes in cytokinin activity have been detected in susceptible barley and wheat cultivars after the onset of powdery mildew spore production (6 - 10 dpi) (Vizárová, 1973, 1974b, 1979, 1987; Vizárová *et al.*,1988). Before infection, both resistant and susceptible cultivars were shown to have cytokinin activity which co-chromatographed with zeatin. At 6 dpi susceptible cultivars were found to contain, in addition to zeatin, *iso*-pentenyladenine (2iP) and its derivatives, whereas the resistant cultivars only had zeatin activity. Vizárová (1987) supposed that,

during sporulation the fungus produces 2iP and its derivatives in the susceptible host plant.

Very few studies have examined the changes in endogenous cytokinins of wheat cultivars infected with stem rust. Sziraki *et al.* (1976) found that the rust-induced increase in cytokinin activity was greater in the susceptible cultivar. The susceptible cultivar Little Club (*Triticum compactum*) was seen to have a slightly higher level of cytokinin activity than the resistant cultivar Vernal (*Triticum dicoccum*) before inoculation. The differences in genetic backgrounds of the two cultivars could account for these differences in cytokinin levels. An identical pattern of changes in endogenous cytokinin levels as recorded for powdery mildew infected barley leaves was seen in wheat leaves infected by *Puccinia graminis* f.sp. *tritici* (Vizárová *et al.*, 1988; Vizárová *et al.*, 1986). In a susceptible and a moderately resistant cultivar, a new zone of cytokinin activity was detected in rust-infected leaf material, whereas no new zone was detected in resistant cultivars. The new zone detected in the wheat cultivars is the same as that detected in powdery mildew susceptible barley cultivars and identified as 2iP and its derivatives by Vizárová (1987).

This literature review has highlighted the possible rôles of cytokinins in plant pathogenesis, and emphasizes the fact that many discrepancies and contradictions appear in the literature. The reports by Vizárová and her co-workers on the possible important role of free endogenous cytokinins in the resistance of cereals against powdery mildew stimulated the present author's interest in the role of these substances in the resistance of wheat to stem rust.

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CHAPTER 1

LEVELS OF CYTOKININS IN SUSCEPTIBLE AND RESISTANT WHEAT-STEM RUST INTERACTIONS

INTRODUCTION

A number of investigations have shown that barley and wheat cultivars resistant to powdery mildew infections have higher levels of endogenous cytokinins before infection than susceptible cultivars, and a close correlation has been found between resistance and cytokinin activity (Kern *et al.*, 1987; Vizárová, 1975b, 1979, 1987; Vizárová & Paulech, 1979). Dried seed material of resistant cultivars of these two cereals was also found to contain higher levels of endogenous cytokinins (Vizárová & Muzikova, 1981; Vizárová & Vozar, 1984), and Vizárová (1987) found that the resistant cultivars of these two cereals have higher cytokinin activity during their entire ontogeny than the susceptible cultivars. These results point to the possible important role of free endogenous cytokinins in the resistance of cereals against powdery mildew (Vizárová, 1987) and stimulated the present interest in the role of endogenous cytokinins in the resistance of wheat to stem rust.

Few studies have examined the changes in endogenous cytokinins of wheat cultivars induced by stem rust infection. However, the pattern of changes detected in stem rust infected wheat leaves was seen to be the same as that in powdery mildew infected barley leaves (Vizárová *et al.*, 1988; Vizárová *et al.*, 1986). Infection of barley with powdery mildew (Vizárová, 1974a, b, 1975b, 1979; Vizárová & Kováčová, 1980; Vizárová & Minarcic, 1974), and wheat with stem rust or powdery mildew (Kern *et al.*, 1987; Sziraki *et al.*, 1976; Vizárová *et al.*, 1988; Vizárová *et al.*, 1986) has been shown to induce an increase in the levels of endogenous cytokinins in both susceptible and resistant cultivars, however the increase in susceptible cultivars was far greater than that in the resistant cultivars.

Qualitative changes in cytokinin activity detected in susceptible cultivars of barley

infected with powdery mildew (Vizárová, 1973, 1974b, 1979, 1987), and susceptible or moderately resistant wheat cultivars infected with stem rust (Vizárová *et al.*, 1986) indicate that, during spore production, these pathogens produce cytokinins in the host plants and in so doing would contribute to the cytokinin pool of the infected plant. Cytokinin production by these pathogens in fully resistant cultivars has not been detected, as these cultivars have the same cytokinin activity as healthy resistant cultivars (Vizárová *et al.*, 1988).

This research was aimed at determining whether wheat cultivars resistant to stem rust have higher levels of endogenous cytokinins than susceptible cultivars, and the potential of cytokinin levels as an indicator of resistance in wheat breeding selection criterion. The work has been restricted to endogenous cytokinin levels of disease-free plants, because for a plant breeder, the ability to carry out disease resistance selections, without the complication of the pathogen, would be a great advantage. The susceptible wheat cultivar Little Club (*Triticum compactum*) and the resistant isogenic line Little Club *Sr25* were selected to minimise differences caused by genetic background. Stem rust race 2SA4 is the most common race in South Africa, and gives an infection type 4 on Little Club, and 2 on Little Club *Sr25* on the Stakman scale (Stakman *et al.*, 1962).

MATERIALS AND METHODS

Two separate experiments were carried out in this study, both aimed at a comparative quantitative and qualitative analysis of the endgenous cytokinins in the leaf and seed material of a susceptible cultivar and an isogenic resistant wheat line of the susceptible cultivar. The general techniques used in this investigation are presented initially, and this is followed by the presentation of the specific methodology employed in each of the two experiments.

Plant material. For the analysis of leaf material, the wheat cultivar Little Club and the isogenic wheat line Little Club *Sr25*, were grown in trays (26.5 by 18.5 by 6.5

cm) of washed river sand in a Conviron at 26°C/16°C, 12 hour/12 hour day/night regime. Primary leaves were harvested 15 days after sowing, flash frozen in liquid nitrogen, packed into polythene bags and stored at -20°C until required. Dried seed material was milled when required.

Extraction of cytokinins from plant material. In both experiments, cytokinins were extracted from 5 g of leaf and 2 g of seed material by homogenising the sample in 100 ml of 80% ethanol and being allowed to stand for 24 hours at 5°C. The homogenates were then filtered through Whatman No. 1 filter paper and the residues were washed with 80% ethanol. The extracts were then concentrated to dryness *in vacuo* at 30°C and resuspended in accordance with the chromatographic technique to be employed.

Chromatographic Techniques:-

Paper chromatography. Extracts were resuspended in 2 ml of 80% ethanol, filtered through 0.45 μm Millipore filters and strip-loaded onto Whatman No. 1 chromatography paper. The constituents were separated by descending chromatography using *iso*-propanol:25% NH_4OH:water (10:1:1 v/v) (PAW) until the solvent front had advanced approximately 30 cms from the origin. Thereafter, the solvent fronts were marked and the chromatograms dried in a drying oven at 25°C for 24 hours. The dry chromatograms were divided into ten equal zones. If at this point, the chromatographed extracts were to be analysed for cytokinin activity, the strip of paper corresponding to each R_f zone was cut up and placed into a 50 ml Erlenmeyer flask and subsequently assayed for cell-division promoting activity using the soybean callus bioassay (Miller, 1963, 1965). The chromatograms were stored at -20°C if further analysis was necessary.

Column chromatography. Column chromatography was used to fractionate extracts so that the cytokinins could be tentatively identified on a basis of co-elution with authentic cytokinin markers.

The technique used was based on that of Armstrong *et al.* (1969). The columns (90 x 2.5 cms) were packed with Sephadex LH-20 which had been swollen in 35% ethanol. They were eluted with 35% ethanol at a flow rate of 15 ml per hour at 20°C. Dried extracts were resuspended in 1 ml 35% ethanol and loaded onto the columns. After elution through the columns, the fractions were combined in 40 ml fractions in Erlenmeyer flasks. Ten ml aliquots of each fraction were assayed for cell division activity using the soybean callus bioassay (Miller, 1963, 1965) and the remainder was dried on a hot plate (30°C) in a stream of air and stored until required for further analysis.

High performance liquid chromatography. High performance liquid chromatography separation of authentic cytokinins, and cytokinins from plant extracts was achieved by reversed phase high performance liquid chromatography (HPLC). The column used was a Hypersil 5 ODS column (250 x 4 mm i.d.) with a flow rate of 1 ml per minute. This was maintained by a 3 500 pounds per square inch single piston reciprocating pump, absorbance was recorded with a Varian variable wavelength monitor at 265 nm, which was fitted with an 8 μm flow-through cell. Separation was achieved using a Varian 5 000 liquid chromatogram and the data output recorded using a Vista 4 000 data system.

Partially purified extracts obtained after paper chromatography or Sephadex LH-20 column chromatography were redisolved in methanol and filtered through a 0.22 μm Millipore filter. 100 μl aliquots were injected into the chromatograph. At the start of the programme the mobile phase consisted of methanol:0.2M acetic acid buffered to pH 3.5 using triethylamine (5:95) at a flow rate of 1 ml per minute and a column temperature of 30°C. Aliquots of 1 or 2 ml from each sample run were collected, air dried and then assayed for cell division activity.
In order to obtain the elution times of various endogenous cytokinins occurring in plant material, authentic cytokinin standards were run through the same column using the same programme.

Soybean Callus Bioassay. The soybean cotyledonary callus bioassay was used

to determine cytokinin-like activity of plant extracts separated on paper, column and HPLC chromatograms. Of the various cytokinin assay systems in use, tissue culture bioassays are regarded as being the most sensitive. According to Van Staden & Davey (1979), the soybean (*Glycine max* L. cv. Acme) callus bioassay is probably the best tissue culture assay to use because it exhibits a linear relationship between response and concentration over a wide range of cytokinin concentrations. Advantages of this bioassay are that microbial growth is eliminated and also that no natural cytokinins have been detected in soybean callus maintained on kinetin (Van Staden & Davey, 1977).

The procedure described by Miller (1963, 1965) was followed in obtaining callus from the cotyledons of soybean. This was maintained by three-weekly subculture.

Four stock solutions were prepared and the nutrient medium made up as outlined in Appendix 1.1. Twenty ml of medium was added to 50 ml Erlenmeyer flasks containing 0.2 g of agar, non-absorbant cotton wool bungs were used to stopper the flasks and these were then covered with aluminium foil. The flasks were autoclaved at a pressure of 1.05 Bars for twenty minutes before being transferred to a sterile transfer cabinet. The inside of the transfer cabinet was then sprayed with 100% Thymol and left to dry for six hours. Thereafter, three pieces of soybean stock callus, each of approximately 10 mg, were placed on the medium in each flask. The flasks were then incubated in a growth room where a constant temperature (26°C ± 2°C) was maintained. The three pieces of callus were massed simultaneously after twenty-eight days. The amount of callus growth relative to the control value was plotted. Kinetin standards were included with each bioassay.

Experiment 1: Determination of endogenous cytokinin levels in a susceptible wheat cultivar and a resistant isogenic wheat line, and the tentative identification of these cytokinins using paper and high performance liquid chromatography

In this experiment, the cytokinins in the plant material extracts were initially

separated using the paper chromatographic technique described previously.

Each dried chromatogram was cut in half, one half of which was used for a soybean callus bioassay immediately, the other half of which was stored at -20°C, to be used for HPLC and then soybean callus bioassay once the bioassay results of the first half were known.

Each of the remaining half chromatograms were cut at the 0.5 R_f value, cut up into 100 ml Erlenmeyer flasks and the cytokinins eluted from the paper by two, two hour washes in 50 ml 80% redistilled ethanol. The extracts were then evaporated to a smaller volume in 500 ml Buchi flasks, transferred to small pear shaped Buchi flasks and evaporated to dryness. The extracts were then resuspended in 400 μl HPLC grade methanol, filtered and 100 μl injected into the chromatograph as previously described. Aliquots of 1 ml per minute over a period of 90 minutes were collected from each sample run. These were washed into 25 ml Erlenmeyer flasks, dried on a hot plate at 30°C in a stream of air and then prepared for the soybean callus bioassay. The remaining 300 μl of each sample was dried and stored in Eppendorf tubes.

Experiment 2: Determination of endogenous cytokinin levels in a susceptible wheat cultivar and a resistant isogenic wheat line, and the tentative identification of these cytokinins using column and high performance liquid chromatography

In experiment 2, the cytokinins in the four plant material extracts were initially separated using the column chromatography technique described.

Soybean callus bioassays were carried out on 10 ml of each 40 ml fraction for each of the four samples, the remainder was evaporated to dryness and stored until the bioassay results were known. Two repititions of each sample were analysed.

The dried extracts from the Sephadex columns (forty flasks for each of eight

samples) were resuspended in two washes of 5 ml 80% redistilled ethanol with the five groups of fractions being pooled and dried under vacuum. The extracts were then resuspended in 1 ml 80% ethanol, passed through a 0.22 μm Millipore filter into a clean glass vial. The extracts were then dried *in vacuo* using a vacuum centrifuge and then resuspended in 300 μl of 80% HPLC grade methanol. The extracts were then filtered again through a 0.22 μm Millipore filter and loaded onto the HPLC column as described previously. The fraction collector was set to collect 2 ml fractions every two minutes for ninety minutes. These fractions were then washed into 25 ml Erlenmeyer flasks, dried on a hot plate (30°C) in a stream of air and then prepared for assay using the soybean callus bioassay. The remaining 200 μl of extract was reduced to dryness in the vial *in vacuo* on a vacuum centrifuge and stored at 10°C until required.

RESULTS

For the sake of clarity the following abbreviations have been used in the text, tables and figures:- Z = zeatin, ZR = ribosylzeatin, ZG = glucosylzeatin,
Ade = adenine, Ado = adenosine, ZOG = zeatin-O-glucoside, Z9G = zeatin-9-glucoside, tZ = *trans*-zeatin, DHZ = dihydrozeatin, DHZOG = dihydrozeatin-O-glucoside, tZR = *trans*-ribosylzeatin, DHZR = dihydroribosylzeatin,
2iP = *iso*-pentenyladenine, 2iP9G = *iso*-pentenyladenine-9-glucoside,
iPA = *iso*-pentenyladenosine.

Soybean callus yield (g/flask) obtained for the kinetin standards (at concentrations of 0, 1, 10 an 50 μg/l) of each bioassay run are indicated on each graph of cytokinin-like activity. (See also APPENDIX 1.2).

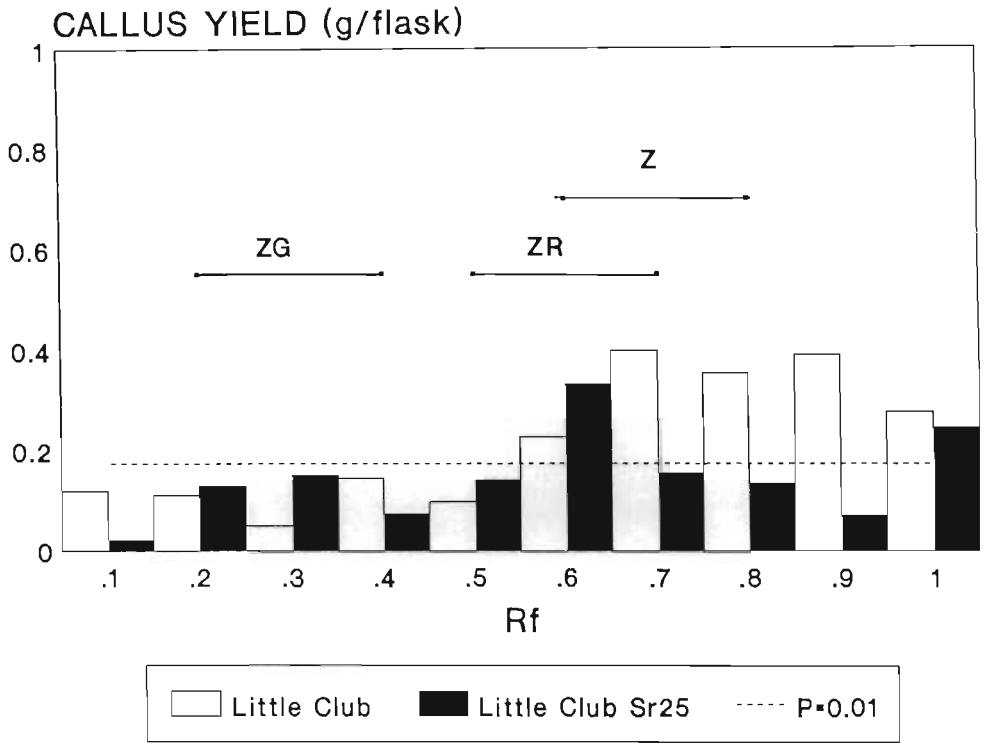
Experiment 1

Paper chromatography

The R_f values of authentic cytokinin markers are superimposed on the figures of cytokinin-like activity detected in the plant material using the soybean callus bioassay.

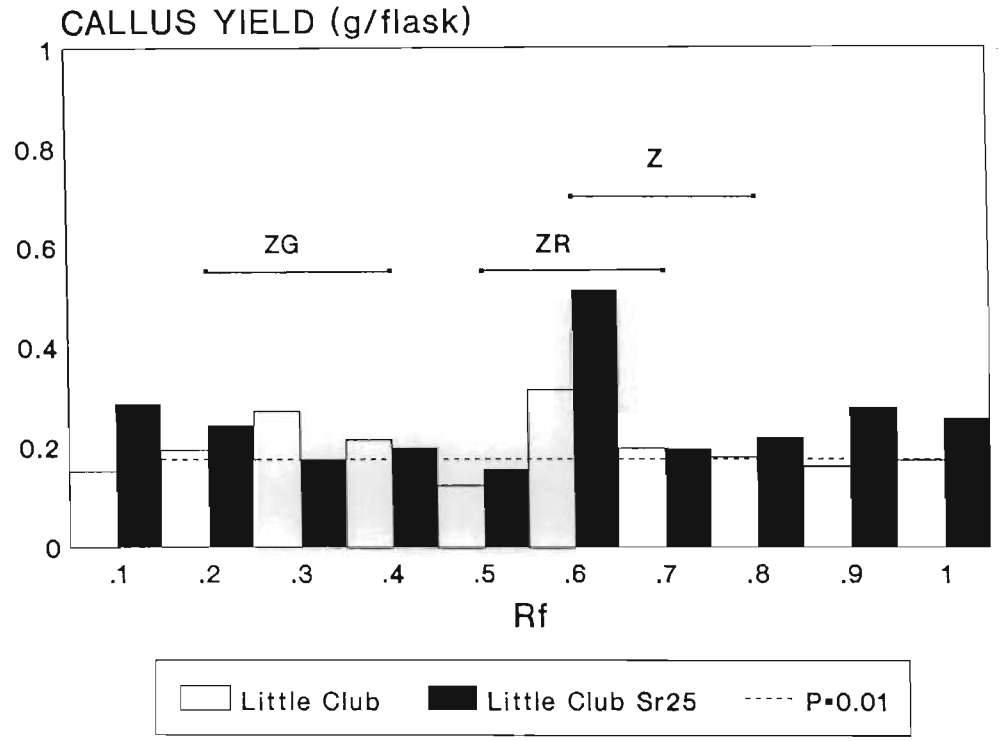
Leaf material (Fig. 1.1.a, see also APPENDIX 1.3 Table 1)
Two peaks of cytokinin-like activity were detected in the extract of the resistant material, namely a peak corresponding to R_f 0.6 which co-chromatographed with Z and ZR, and a peak corresponding to R_f 1.0 which did not co-chromatograph with any of the markers used. In the extract of the susceptible material, significant peaks were detected at R_fs 0.6, 0.7, 0.8, 0.9 and 1.0. The peak at R_f 0.6 detected in the resistant material was larger than the corresponding peak in the susceptible material.

Seed material (Fig. 1.1.b, see also APPENDIX 1.3 Table 1)
In the resistant material, a number of peaks of biological activity were detected, namely peaks at R_f 0.1, 0.2, 0.4, 0.6, 0.7, 0.8, 0.9 and 1.0. In the susceptible material slow-moving peaks recovered at R_f 0.2, 0.3 and 0.4 which co-chromatographed with ZG were detected, as was a faster-moving peak which corresponded to R_fs 0.6 and 0.7. The peaks corresponding to R_f 0.3 and 0.4 co-chromatographed with ZG, and the results indicate higher levels of ZG in susceptible than resistant material. The peaks corresponding to R_f 0.6 and 0.7 in both cultivars co-chromatographed with ZR and Z, and the results indicate that the resistant line has higher levels of these two cytokinins than does the susceptible cultivar.



Z = zeatin; ZR = ribosylzeatin; ZG = glucosylzeatin

Fig. 1.1a Soybean callus bioassay of 2.5g Little Club and Little Club *Sr25* primary leaf material. Cytokinins were separated on paper with *iso*-propanol:25% NH4OH:water (10:1:1 v/v). The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 1).



Z = zeatin; ZR = ribosylzeatin; ZG = glucosylzeatin

Fig. 1.1b Soybean callus bioassay of 2.5g Little Club and Little Club *Sr25* seed material. Cytokinins were separated on paper with *iso*-propanol:25% NH4OH:water (10:1:1 v/v). The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 1).

High Performance Liquid Chromatography

The elution times of the authentic cytokinin markers are listed in Appendix 1.2 and have been superimposed upon the figures of cytokinin-like activity detected using the soybean callus bioassay. One sample (Little Club *Sr25* R_f 0.5 - 1.0) was lost while preparing it for HPLC separation and because of this, the results for the leaf material were used for a qualitative investigation of the cytokinin activity, whereas data from the seed material were used for both qualitative and quantitative investigation of cytokinin activity.

Leaf material (See also APPENDIX 1.3 Table 2)
Biological activity from R_f 0.1 - 0.5 and R_f 0.6 - 1.0 are presented in Figs. 1.1c and 1.1d respectively. Fig. 1.1e represents the pooled data from the two halves of each chromatogram of leaf material for both wheat selections. As noted previously, data for Little Club *Sr25* R_f 0.5 - 1.0 have not been included. Material from the resistant line gave significant biological activity at retention times which corresponded to a number of the authentic cytokinin markers, namely Ado, tZ, DHZ, tZR, DHZR, 2iP and iPA. The susceptible cultivar showed biological activity at retention times at which the following authentic cytokinins co-eluted, Ade, Z9G, DHZ, tZR, DHZR, 2iP9G, 2iP and iPA. Both resistant and susceptible leaf material also showed peaks of biological activity at retention times for which cytokinin markers had not been used.

Seed material (See also APPENDIX 1.3 Table 3)
Biological activity from R_f 0.1 - 0.5 and R_f 0.6 - 1.0 are presented in Figs. 1.1f and 1.1g respectivity. Fig. 1.1h represents the pooled data from the two halves of each chromatogram of seed material for both cultivars. Seed material of the resistant line showed a number of peaks of biological activity at retention times which co-elution with the following authentic cytokinins, Ade, tZ, DHZ, and 2iP9G. The seed material from the susceptible cultivar showed significant biological activity at retention times at which DHZR and 2iP9G co-eluted. Two peaks of biological activity are seen in

susceptible seed material at retention times of 40 to 50 minutes, these did not co-elute with any of the markers used.

In Table 1.1, the total cytokinin-like activity (calculated from pooled data) in the seed material has been converted to kinetin equivalents (KE). The seed material of the resistant line is seen to have significantly greater biological activity that the susceptible cultivar.

Table 1.1 The total cytokinin-like activity in one gram of Little Club and Little Club *Sr25* material. Activity detected after HPLC separation which was significantly different from the controls is expressed as kinetin equivalents (KE)

	Little Club	Little Club *Sr25*
Seed material	42.05 KE	74.64 KE

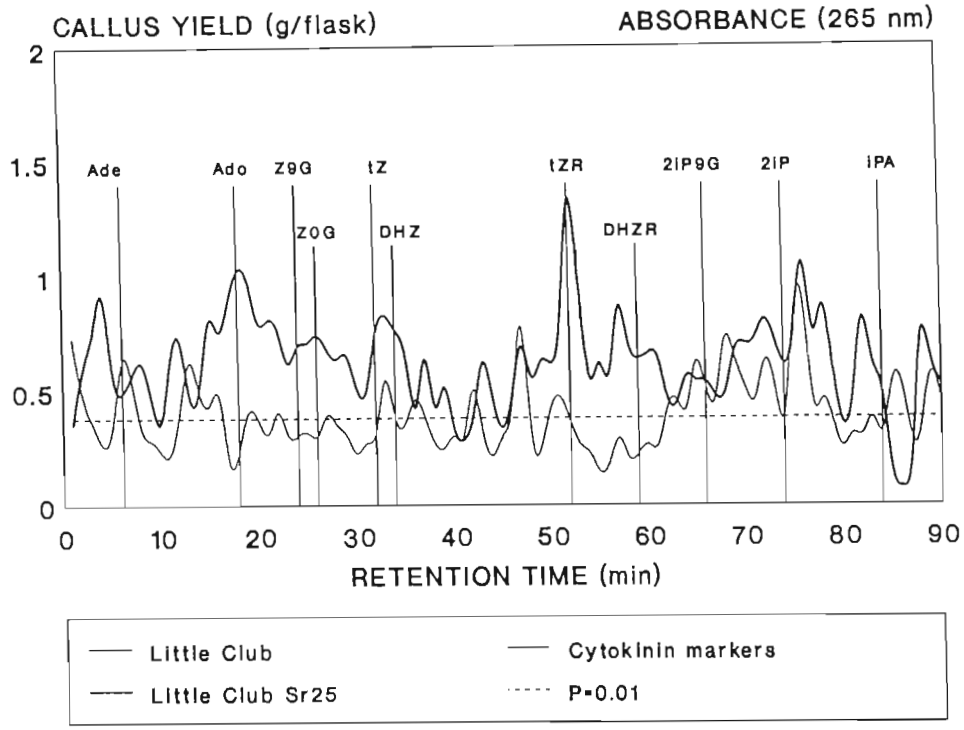
Experiment 2

Column chromatography

The elution volumes of authentic cytokinin markers have been superimposed on the figures of cytokinin-like activity detected in the plant material using the soybean callus bioassay.

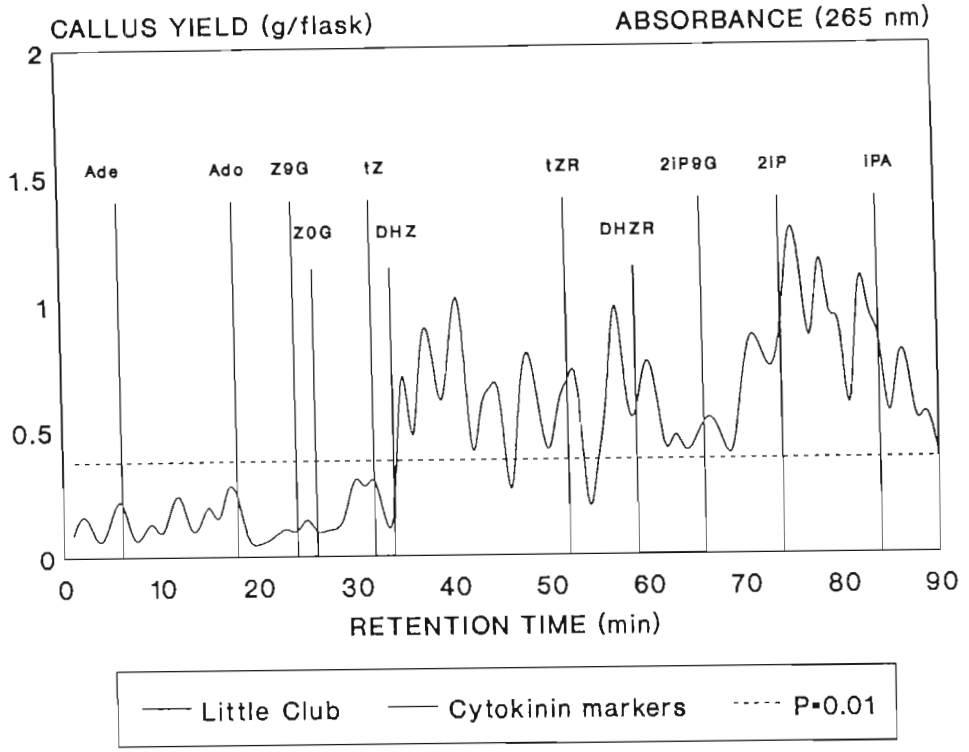
Leaf material (See also APPENDIX 1.4 Table 1)

A number of distinct peaks of biological activity were detected in the Sephadex column eluate of resistant leaf material (Fig. 1.2a). The first peak occurred at an elution volume of 40 - 120 ml and did not co-elute with any of the cytokinin markers used, the second peak had an elution volume of 360 - 480 ml and co-eluted with ZG, the third peak had an elution volume of 640 - 680 ml and co-eluted with Z, the fourth peak had an elution volume of 800 - 960 ml and co-eluted with iPA, the fifth peak occurred at 1120 ml and co-eluted with 2iP, the sixth peak had an elution volume of 1320 ml and did not co-elute with any of the cytokinin markers used.



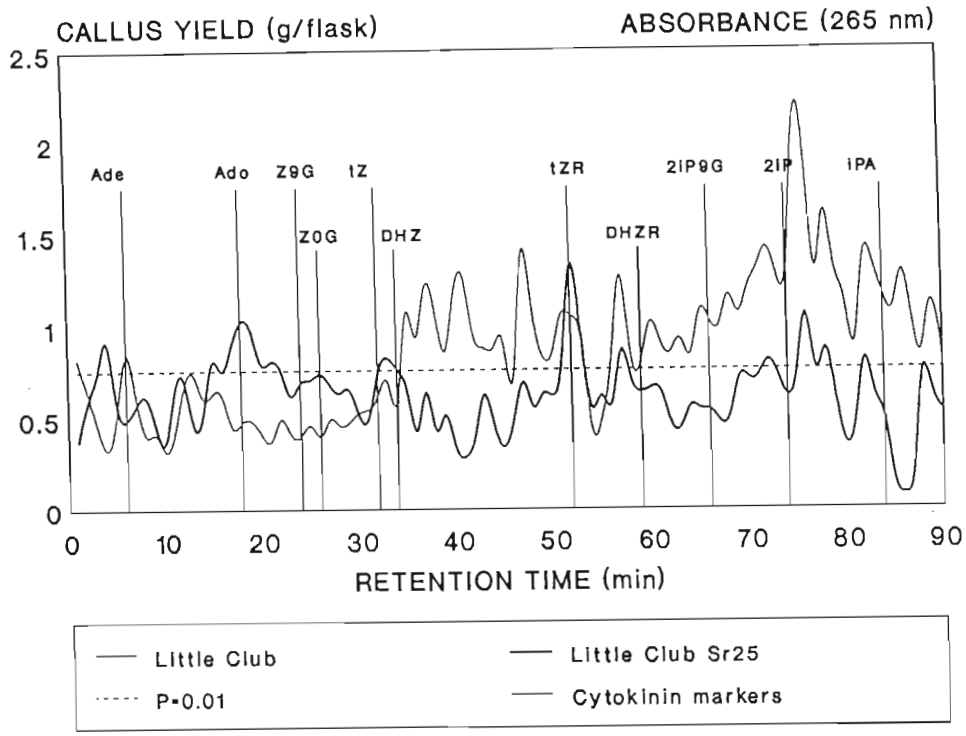
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1c Soybean callus bioassay of cytokinin-like activity in R_f 0.1 - 0.5 (Fig. 1.1a) of Little Club and Little Club *Sr25* primary leaf material after HPLC separation. The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. This represents activity in 0.3125g of material. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 2).



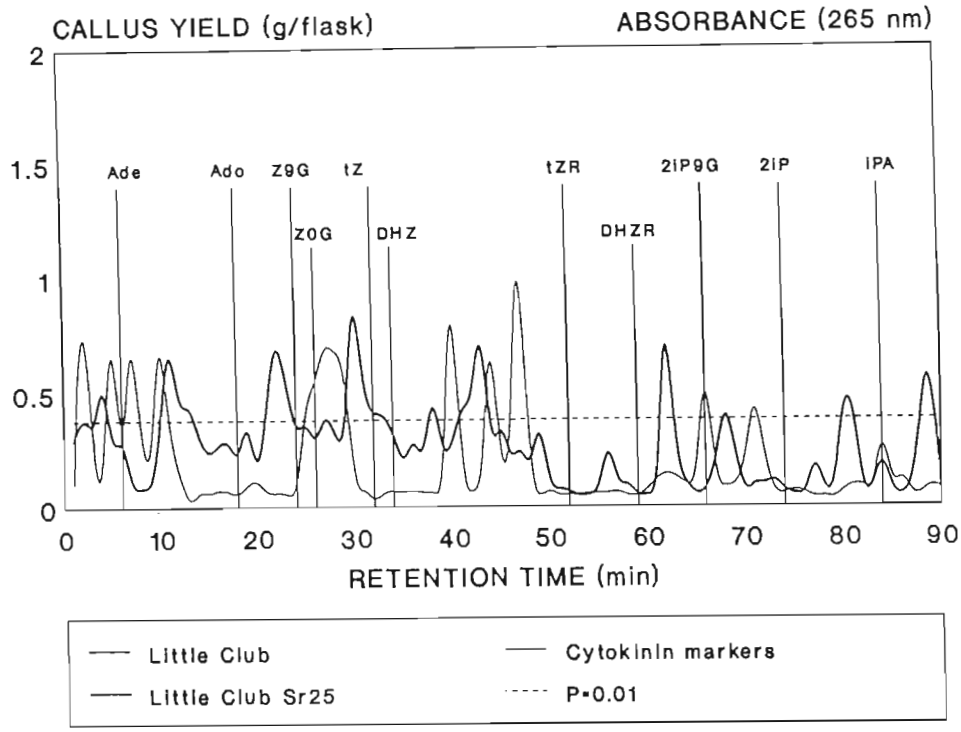
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = trans-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1d Soybean callus bioassay of cytokinin-like activity in R_f 0.6 - 1.0 (Fig. 1.1a) of Little Club primary leaf material after HPLC separation. The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. This represents activity in 0.3125g of material. The dotted line indicates the confidence limit at the level P = 0.01. The sample containing R_f 0.6 - 1.0 of Little Club *Sr25* was lost while preparing it for HPLC analysis. (See also APPENDIX 1.3 Table 2).



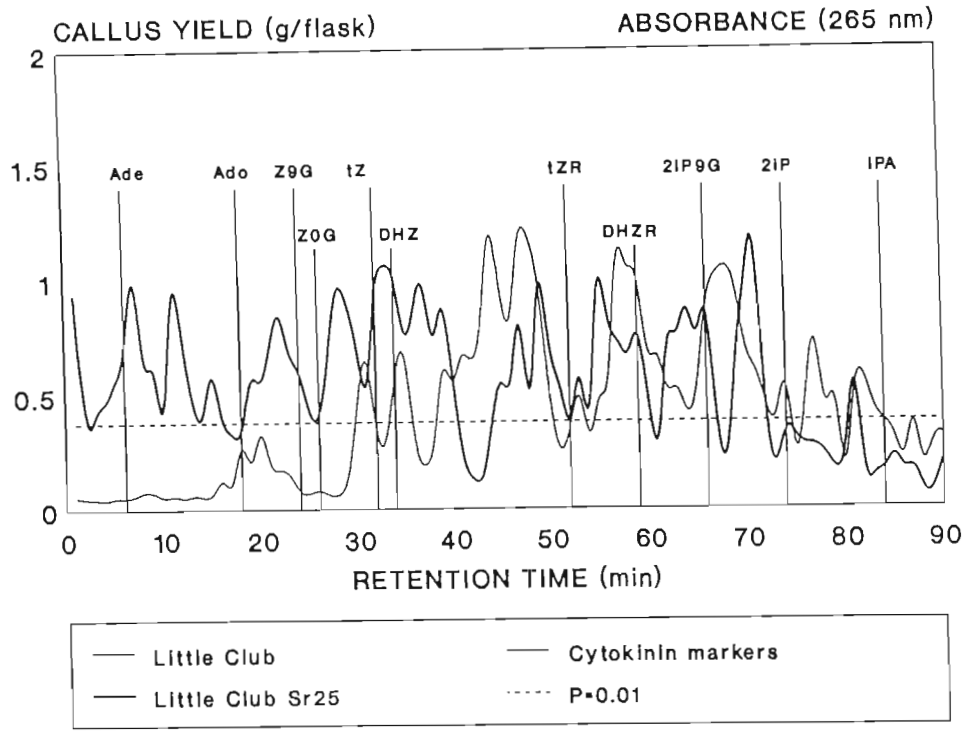
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-O-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1e Pooled cytokinin-like activity detected in Little Club and Little Club *Sr25* primary leaf material (Figs. 1.1c and 1.1d). The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. This represents activity in 0.3125g of material. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 2).



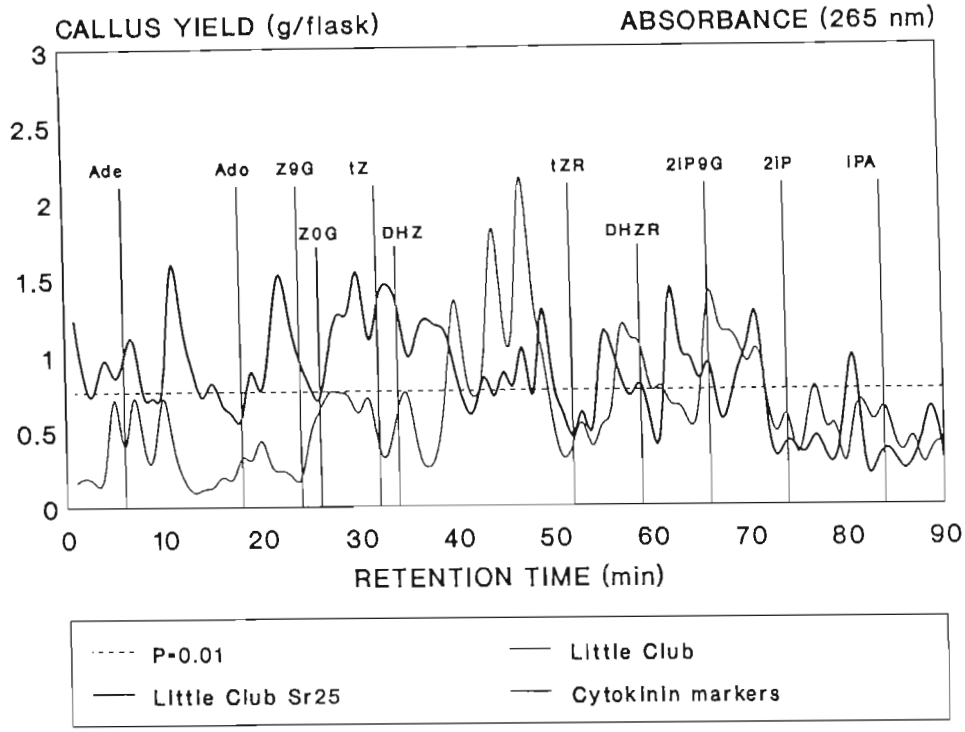
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-O-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1f Soybean callus bioassay of cytokinin-like activity in R_f 0.1 - 0.5 (Fig. 1.1b) of Little Club and Little Club *Sr25* seed material after HPLC separation. The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. This represents activity in 0.125g of material. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 3).



Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1g Soybean callus bioassay of cytokinin-like activity in R_f 0.6 - 1.0 (Fig. 1.1b) of Little Club and Little Club *Sr25* seed material after HPLC separation. The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. This represents activity in 0.125g of material. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 3).



Ade = adenine; Ado = adenosine; Z0G = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZ0G = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.1h Pooled biological activity detected in Little Club and Little Club *Sr25* seed material (Figs. 1.1f and 1.1g). The elution times of authentic cytokinin markers (as determined by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.3 Table 3)

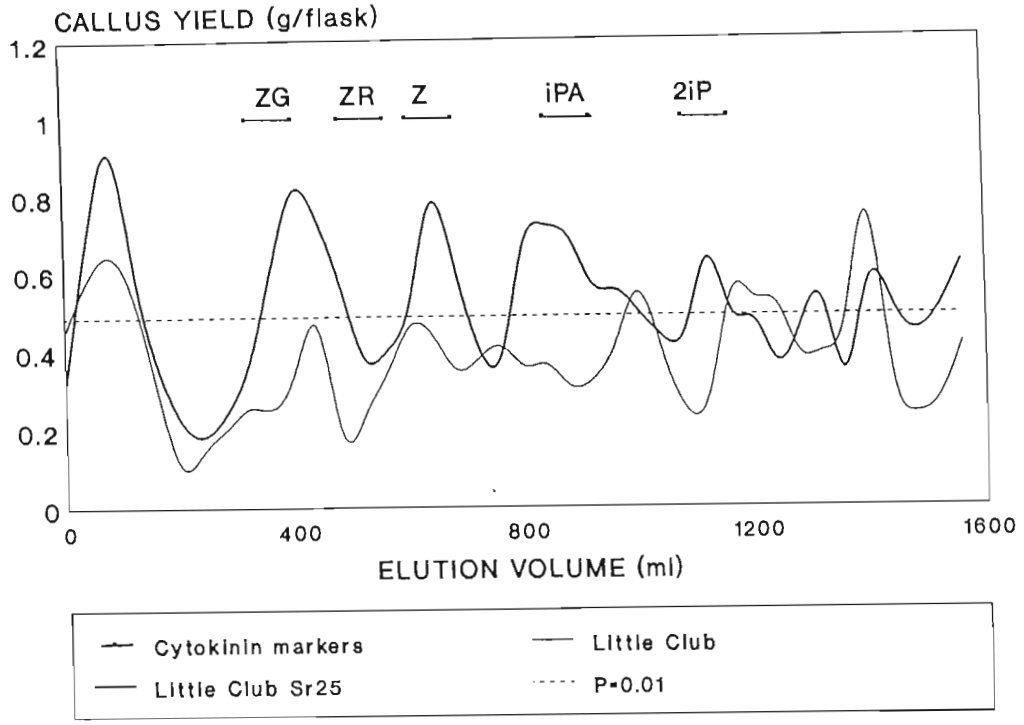
Fewer significant peaks of activity were detected in the Sephadex column eluate of the susceptible leaf material (Fig. 1.2a). The first peak occurred at an elution volume of 40 - 120 ml, the second peak had an elution volume of 1000 ml and co-eluted with iPA, the third peak had an elution volume of 1160 ml and co-eluted with 2iP, the fourth peak had an elution volume of 1400 ml. The first and fourth peaks of biological activity detected in this material did not co-elute with any of the markers used.

Seed material (See also APPENDIX 1.4 Table2)

Three peaks of biological activity were detected in resistant seed material (Fig. 1.2b). The first peak had an elution volume of 480 ml and co-eluted with ZR, the second peak and third peaks had elution volumes of 1000 - 1040 ml and 1280 ml respectively, and did not co-elute with any of the markers used.

Five peaks were detected in susceptible seed material (Fig. 1.2b). The first peak had an elution volume of 80 - 120 ml but did not co-elute with any of the cytokinin markers used, the second peak had an elution volume of 320 ml and co-eluted with ZG, the third peak had an elution volume of 520 -600 ml and co-eluted with ZR, the fourth peak had an elution volume of 680 - 760 ml and co-eluted with Z, the fifth peak had an elution volume of 1200 -1280 ml and did not co-eluted with any of the markers used.

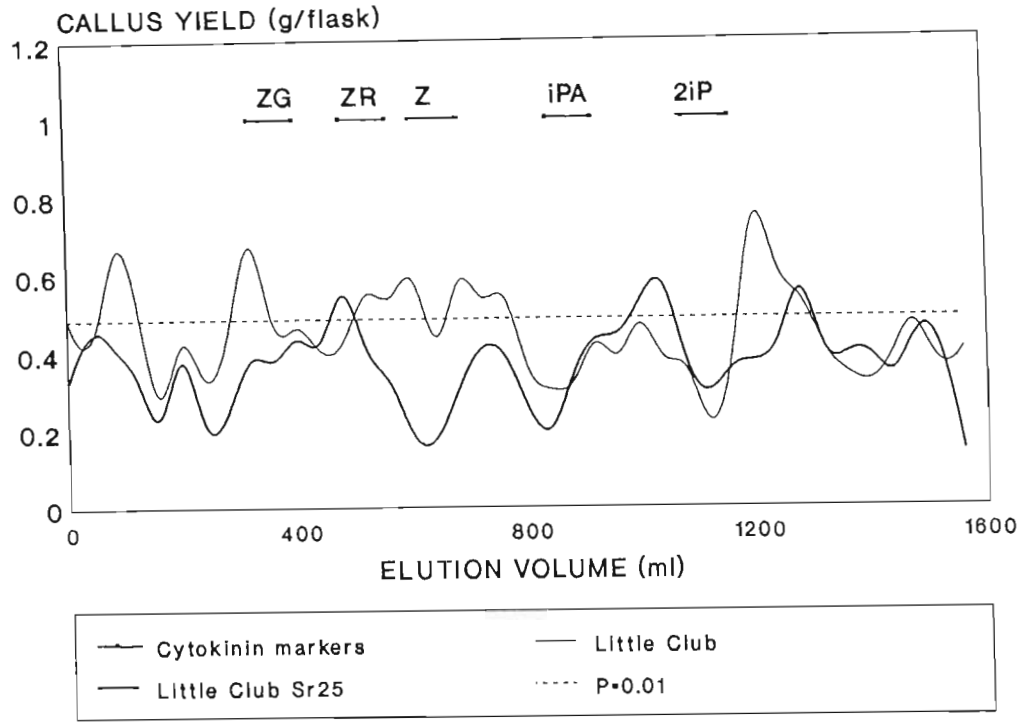
To obtain a better understanding of the role of cytokinins in resistance, the levels of cytokinin activity in leaf and seed material were considered and were expressed as kinetin equivalents (KE). The information is presented in Table 1.2. The results indicate that the leaf material of the resistant line has a greater level of cytokinin activity than the susceptible cultivar, however, the seed material of the susceptible cultivar has higher levels of cytokinin activity than the resistant line.



ZG = glucosylzeatin; ZR = ribosylzeatin; Z = zeatin; iPA = *iso*-pentenyladenosine; 2iP = *iso*-pentenyladenine

Fig. 1.2a Soybean callus bioassay* of cytokinin-like activity detected in 1.25g of Little Club and Little Club *Sr25* primary leaf material, following fractionation on a Sephadex LH-20 column eluted with 35% ethanol. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 1).

* mean biological activity of two replicates



ZG = glucosylzeatin; ZR = ribosylzeatin; Z = zeatin; iPA = *iso*-pentenyladenosine; 2iP = *iso*-pentenyladenine

Fig. 1.2b Soybean callus bioassay* of cytokinin-like activity detected in 0.5g of Little Club and Little Club *Sr25* seed material, following fractionation on a Sephadex LH-20 column eluted with 35% ethanol. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 2).

* mean biological activity of two replicates.

Table 1.2 The total cytokinin-like activity in one gram of Little Club and Little Club *Sr25* material. Activity detected after Sephadex LH-20 separation which was significantly different from the controls is expressed as kinetin equivalents (KE)

	Little Club	Little Club *Sr25*
Primary leaf	1.20 KE	3.29 KE
Seed	4.18 KE	1.09 KE

High Performance Liquid Chromatography

The above-mentioned peaks were further analysed by subjecting the remainder of the fractions collected in the first Sephadex extraction to reverse-phase HPLC, after the fractions had been combined into five sub-samples:- [A] 0 - 200 ml, [B] 200 - 520 ml, [C] 520 - 760 ml, [D] 760 - 1000 ml, [E] 1000 -1600 ml. Results of the biological activity detected in each of the five sub-samples, following separation using HPLC are represented in Figs. 1.2c - 1.2n. The elution times of authentic cytokinin markers are listed in Appendix 1.2 and are superimposed on the figures of cytokinin-like activity detected in the plant material using the soybean callus bioassay.

Leaf material (See also APPENDIX 1.4 Table 3 and Table 4)

In sub-sample A, there was no significant biological activity in the resistant or susceptible material (Fig. 1.2c). In sub-sample B, significant biological activity was detected in susceptible (Fig. 1.2d) material at a retention time of six minutes and this co-eluted with Ade. A peak of significant biological activity was detected at a retention time of 74 minutes in sub-sample C of the resistant material (Fig. 1.2e), this co-eluted with 2iP. No biological activity was detected in the susceptible leaf material in sub-sample C (Fig. 1.2e). In sub-sample D of the resistant material (Fig. 1.2f), biological activity was detected at a retention time of 32 - 36 minutes and co-eluted with tZ and DHZ, and at a retention time of 60 minutes, co-eluted with DHZR. Sub-sample D of the susceptible material (Fig. 1.2f) revealed biological activity at retention times 7, 17, 34 - 36, 66 and 76 minutes, these peaks co-eluting with Ade,

Ado, tZ and DHZ, 2iP9G and 2iP respectively. Sub-sample E of the resistant material (Fig. 1.2g) showed biological activity at a retention time of 85 minutes which co-eluted with iPA, whereas the susceptible material (Fig. 1.2g) showed activity at retention times of 17, 36 and 85 minutes, co-eluting with Ado, DHZ and iPA respectively.

Fig. 1.2h is a graphic representation of pooled data from all five sub-samples of leaf material and as such can be directly compared to Fig. 1.2a which involved separation using Sephadex. Pooled resistant leaf material data yielded four peaks of significant biological activity at retention times 28 minutes (co-eluting with Z9G and ZOG); 32 - 36 minutes (co-eluting with tZ and DHZ); 59 minutes (co-eluting with DHZR) and 75 minutes (co-eluting with 2iP). There is a peak of biological activity co-eluting with iPA at an elution time of 85 minutes, however this peak is not significant.

Pooled susceptible leaf material data indicate two peaks of significant biological activity, at retention times 66 minutes (co-eluting with 2iP9G) and 85 minutes (co-eluting with iPA). The ZG peak indicated in the Sephadex separation is not preseent in the HPLC separation.

Seed material (See also APPENDIX 1.4 Table 5 and Table 6)
The biological activity recorded for seed material fractions A, B, C, D and E are presented in Figs. 1.2i - 1.2m. Fig. 1.2n is a graphic representation of pooled data from the seed material of both cultivars. No significant biological activity is indicated in either susceptible or resistant material, and the peaks of activity observed in the sephadex separation of seed material are not detected.

A quantitative analysis of significant biological activity of the pooled data for the two wheat selections is presented in Table 1.3. This indicates that cytokinin activity of resistant leaf material is greater than that of susceptible leaf material. No such calculations could be made for the seed material as there was no significant biological activity in either selection.

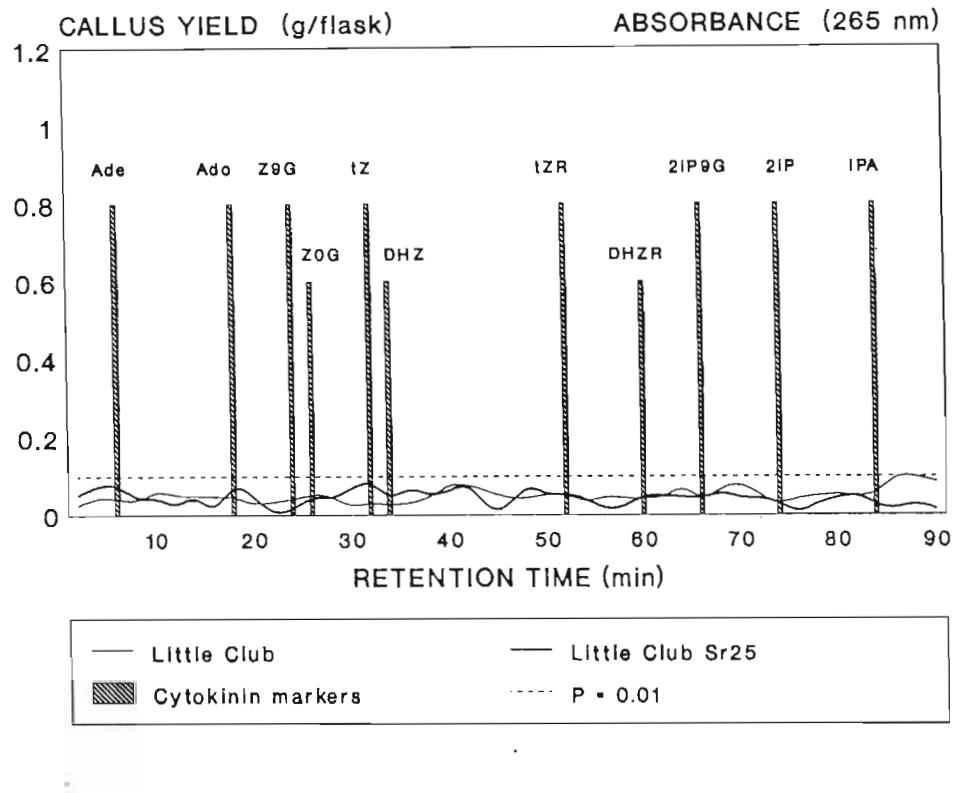
Table 1.3 The total cytokinin-like activity in one gram of Little Club and Little Club *Sr25* leaf material. Activity detected after HPLC separation which was signficantly different from the controls is expressed as kinetin equivalents (KE)

	Little Club	Little Club *Sr25*
Primary leaf	0.86 KE	1.33 KE

DISCUSSION

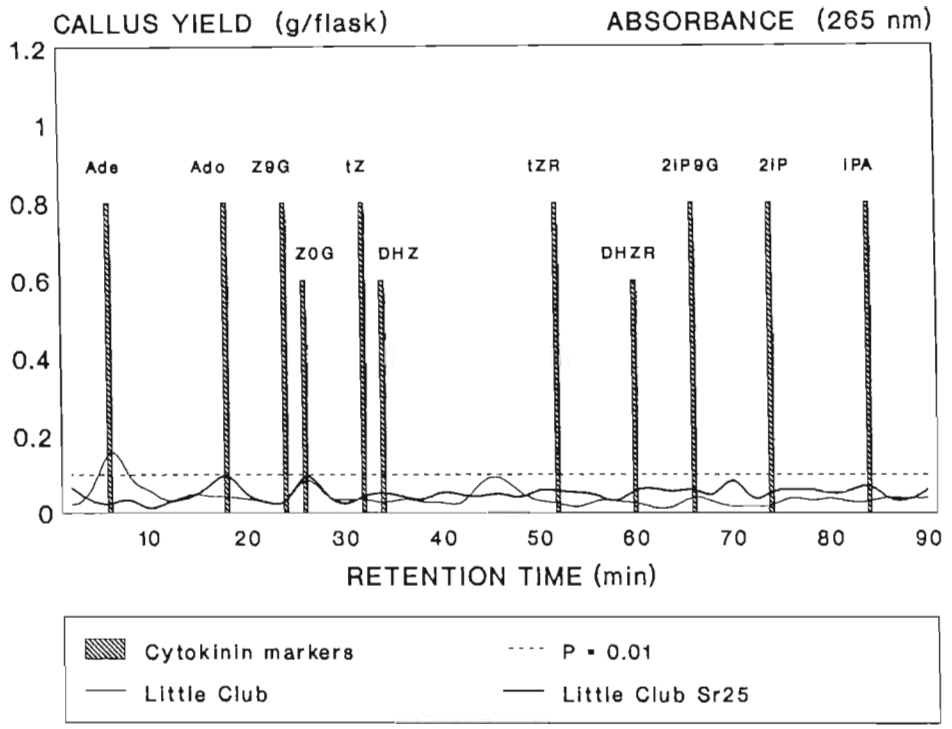
From the literature it appears that the initial level of cytokinin activity is crucial in determining whether a susceptible or resistant cereal/powdery mildew interaction will occur. It has been reported that resistant cultivars have higher initial levels of free endogenous cytokinins in their leaves, in their seeds and indeed, during their entire ontogeny (Kern *et al.*, 1987; Vizárová, 1975b, 1979, 1987; Vizárová & Muzikova, 1981; Vizárová & Paulech, 1979; Vizárová & Vozar, 1984). In Experiment 1 of the present investigation, paper chromatography and HPLC techniques revealed cytokinin-like activity in both leaf and seed material of both the resistant and susceptible wheat selections. The total cytokinin activity of seed material from the resistant line was found to be greater than that of the susceptible cultivar. Sephadex separation of seed material in Experiment 2, however, indicated that the total cytokinin activity of the resistant cultivar was lower than that recorded for the susceptible cultivar. HPLC separation of sub-samples collected after Sephadex separation indicated that neither cultivar had significant cytokinin-like activity in their seed material.

Both the Sephadex and HPLC extraction results of Experiment 2 indicate that leaf material of the resistant line has a higher level of total cytokinin activity than the susceptible cultivar. Any differences detected between Little Club and Little Club *Sr25* would be linked to the *Sr25* gene in Little Club *Sr25*, as except for the presence of this gene in Little Club *Sr25*, the two wheat selections are identical.



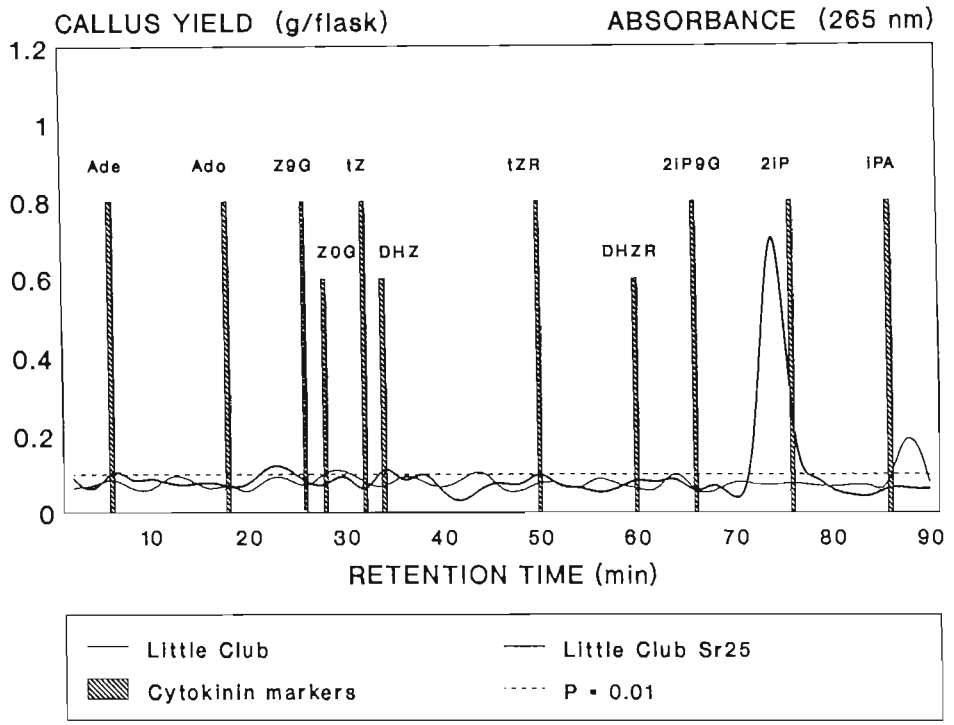
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 4.2c Soybean callus bioassay of Sample A of Little Club and Little Club *Sr25* leaf material, with an elution volume of 0 - 200 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



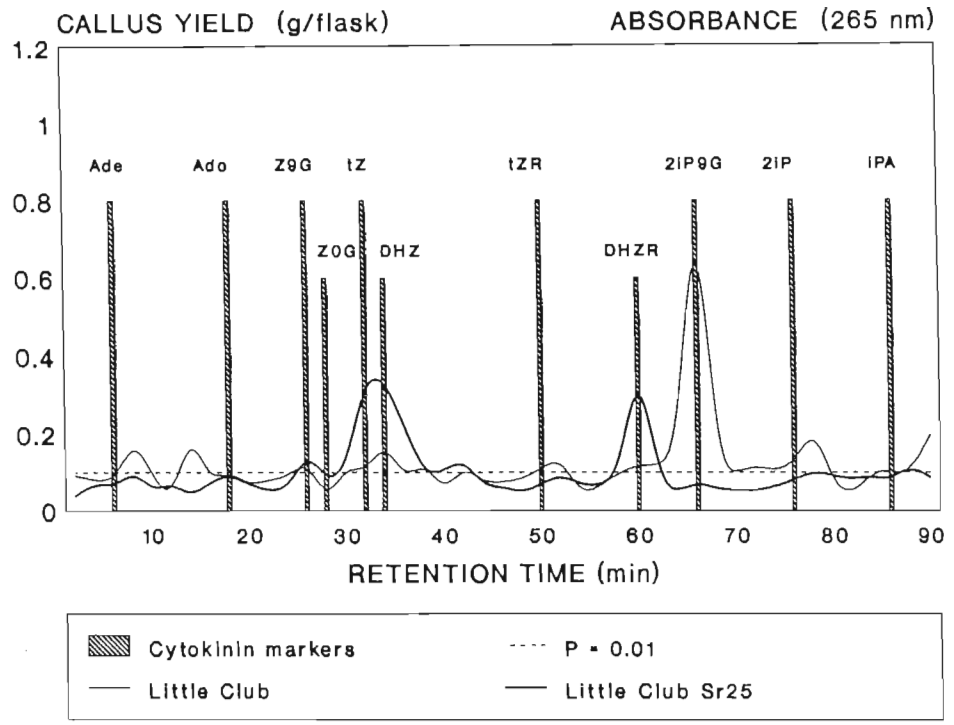
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2d Soybean callus bioassay of Sample B of Little Club and Little Club *Sr25* leaf material, with an elution volume of 200 - 520 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



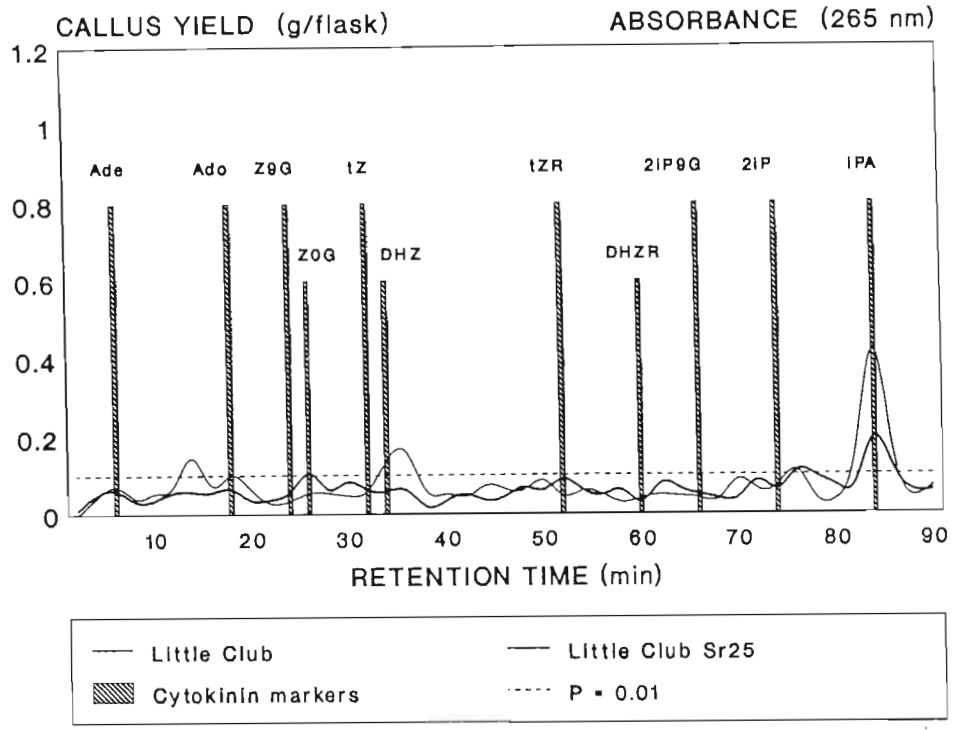
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2e Soybean callus bioassay of Sample C of Little Club and Little Club *Sr25* leaf material, with an elution volume of 520 - 760 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



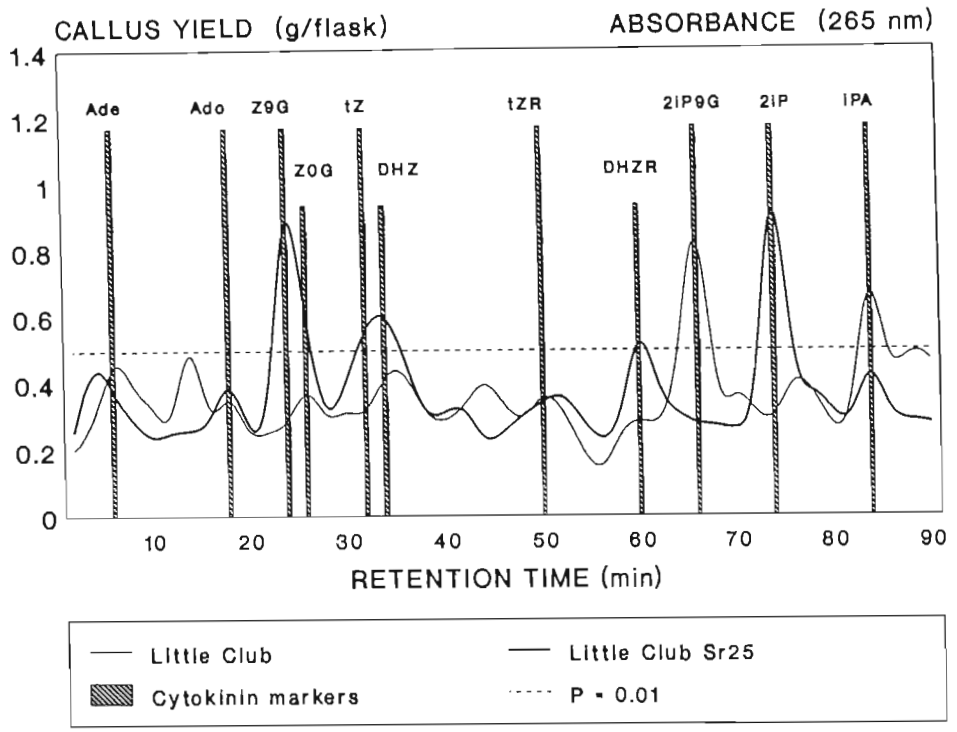
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2f Soybean callus bioassay of Sample D of Little Club and Little Club *Sr25* leaf material, with an elution volume of 760 - 1000 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



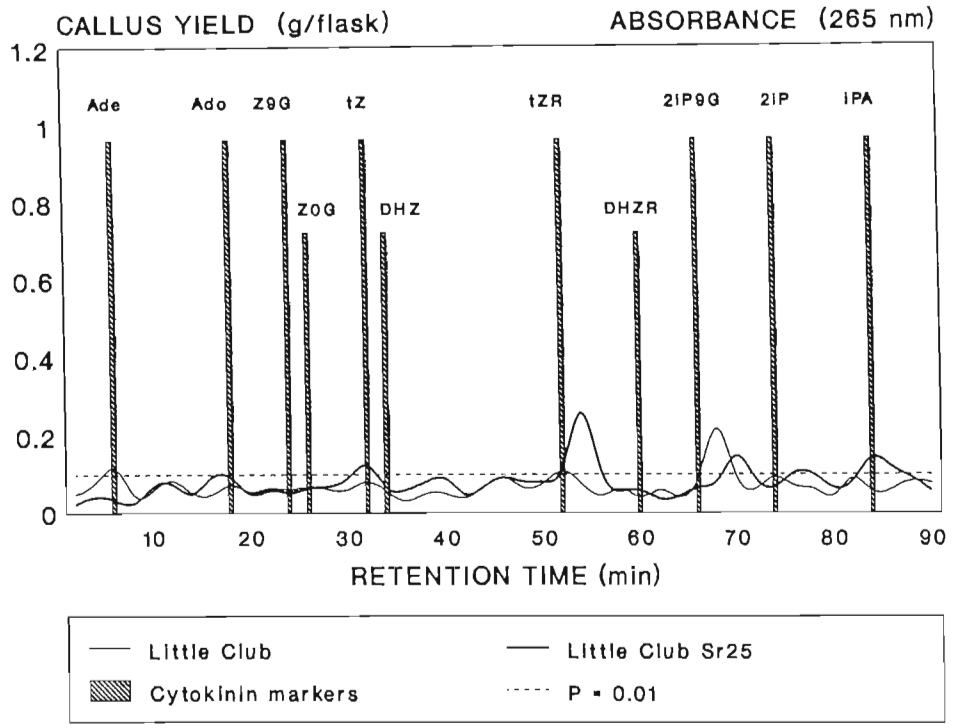
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2g Soybean callus bioassay of Sample E of Little Club and Little Club *Sr25* leaf material, with an elution volume of 1000 - 1600 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



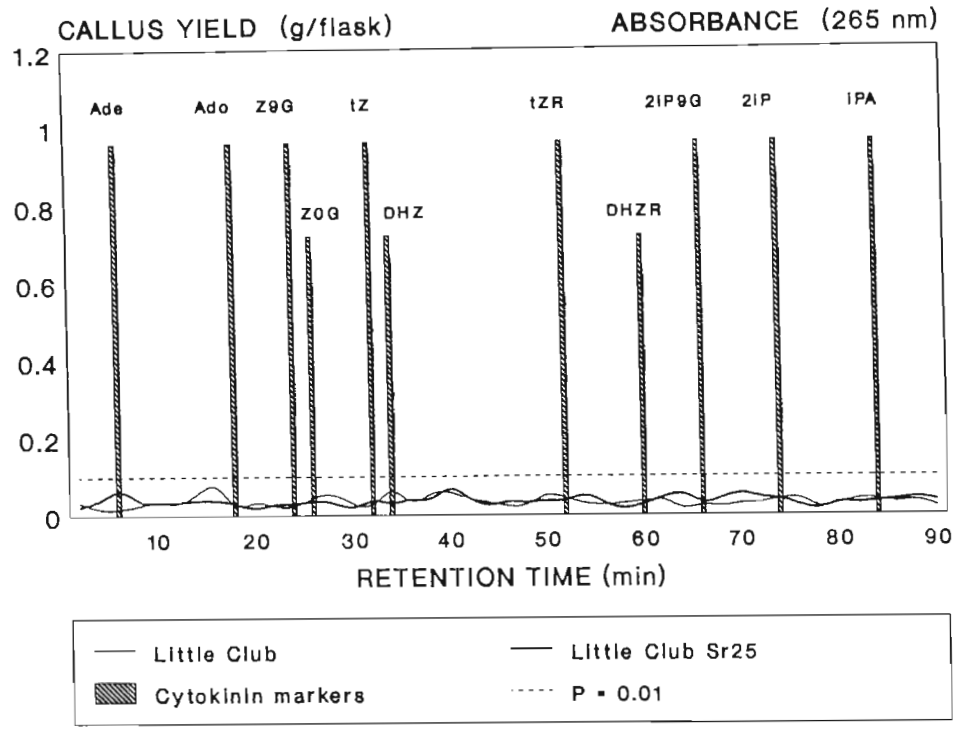
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2h Pooled biological activity detected in Little Club and Little Club *Sr25* primary leaf material (Figs. 1.2c, 1.2d, 1.2e, 1.2f and 1.2g). The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.4 Table 3 and Table 4).



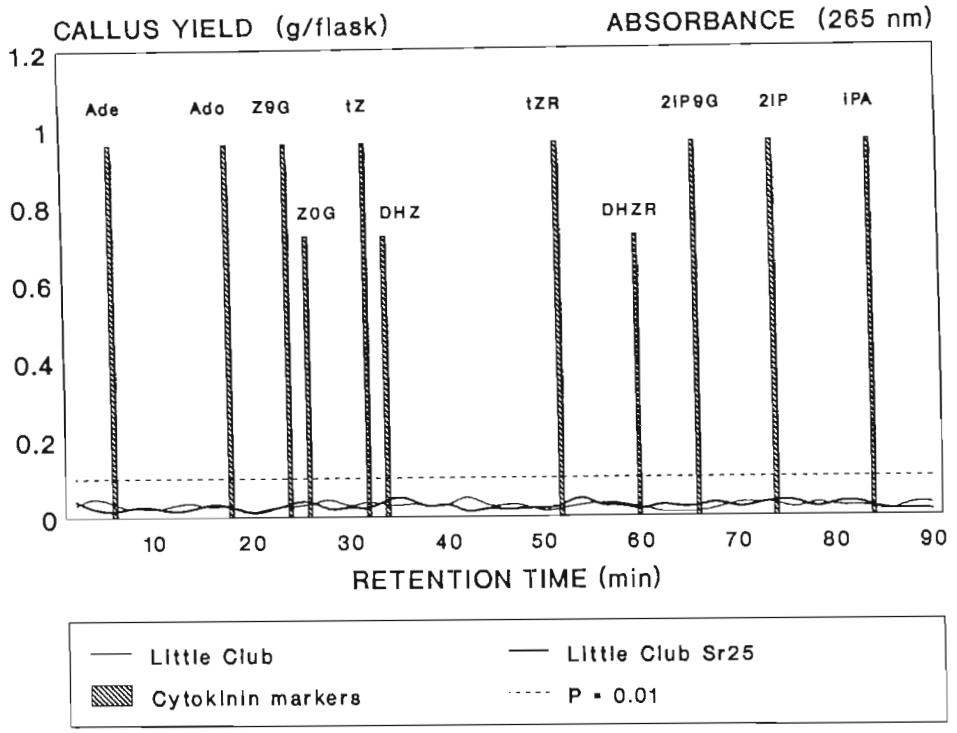
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2i Soybean callus bioassay of Sample A of Little Club and Little Club *Sr25* seed material, with an elution volume of 0 - 200 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).



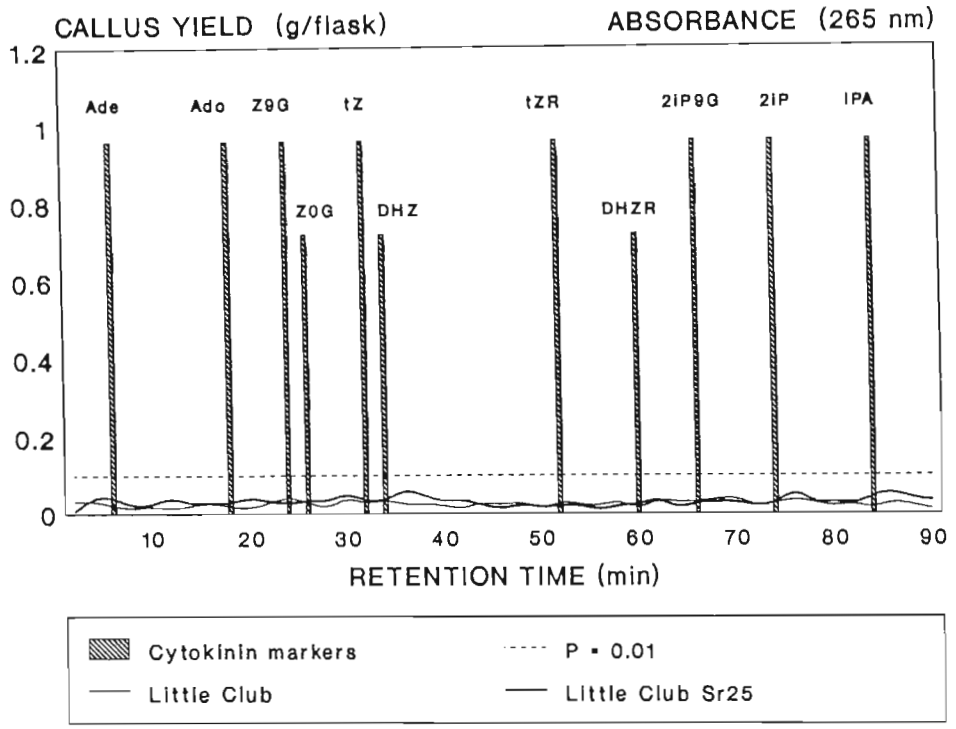
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2j Soybean callus bioassay of Sample B of Little Club and Little Club *Sr25* seed material, with an elution volume of 200 - 520 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).



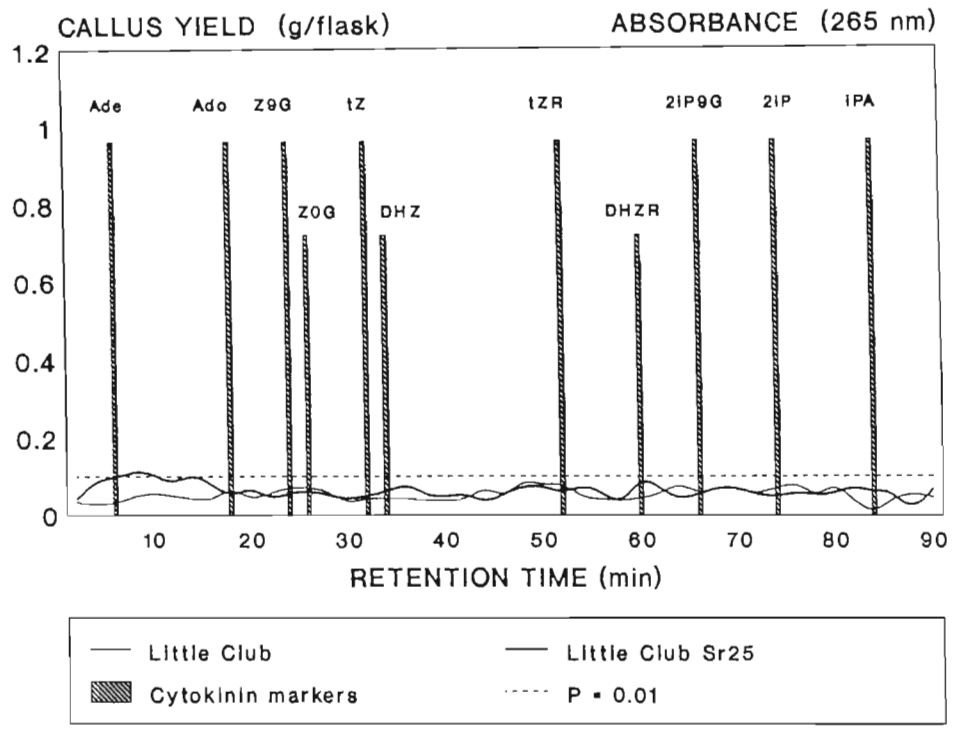
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2k Soybean callus bioassay of Sample C of Little Club and Little Club *Sr25* seed material, with an elution volume of 520 - 760 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).



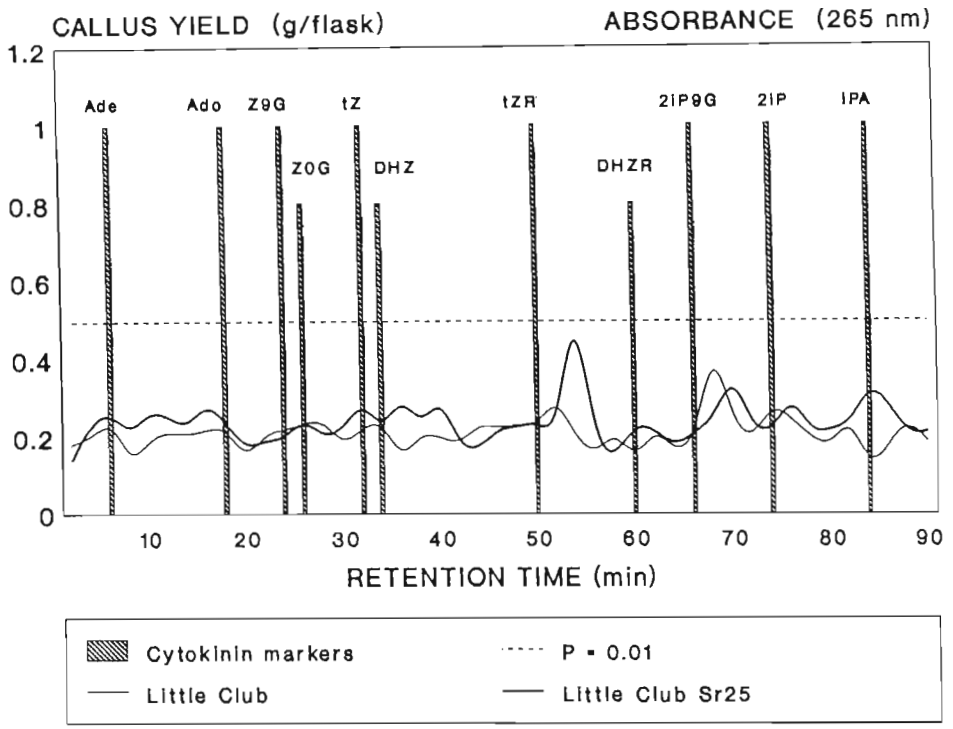
Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2l Soybean callus bioassay of Sample D of Little Club and Little Club *Sr25* seed material, with an elution volume of 760 - 1000 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).



Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2m Soybean callus bioassay of Sample E of Little Club and Little Club *Sr25* seed material, with an elution volume of 1000 - 1600 mls on a Sephadex LH-20 column, which was subjected to HPLC analysis. The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limits at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).



Ade = adenine; Ado = adenosine; ZOG = zeatin-O-glucoside; Z9G = zeatin-9-glucoside; tZ = *trans*-zeatin; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-0-glucoside; tZR = *trans*-ribosylzeatin; DHZR = dihydroribosylzeatin; 2iP = *iso*-pentenyladenine; 2iP9G = *iso*-pentenyladenine-9-glucoside; iPA = *iso*-pentenyladenosine; ZR = ribosylzeatin

Fig. 1.2n Pooled biological activity detected in Little Club and Little Club *Sr25* seed material (Figs. 1.2i, 1.2j, 1.2k, 1.2l and 1.2m). The elution times of authentic cytokinin markers (as detected by UV absorbance at 265nm) are superimposed. The dotted line indicates the confidence limit at the level P = 0.01. (See also APPENDIX 1.4 Table 5 and Table 6).

In the resistance reaction, the fungus is seen to infect the host and some level of colonization occurs. It is possible that the high initial cytokinin levels are fungitoxic at the early stages of infection. The high sensitivity of fungi, in early stages of growth, to cytokinins (Michniewicz *et al.*, 1984), and the findings of Vizárová (1987) that zeatin and its derivatives (at 100 μg per 3 cm3) have an absolute inhibitory effect on the growth of *Erysiphe graminis* do, in part, support this proposal.

Cytokinin-like substances have been detected in wheat grains (Bhardwaj & Dua, 1975; Herzog & Geisler, 1977; Jameson *et al.*, 1982; Reda, 1976; Thomas *et al.*, 1978; Wheeler, 1972, 1976). Changes in levels of these substances in developing wheat grains have been demonstrated to follow a set pattern (Jameson *et al.*, 1982), in that activity is barely detectable at ear emergence but increases markedly at pollination. Levels then increase rapidly until four days after anthesis after which an equally rapid loss occurred. No activity could be detected 21 days after ear emergence. Wheeler (1972) also found that wheat grains had negligible amounts of cytokinin activity. A similar pattern of changes in cytokinin activity has been found in maize (Hocart *et al.*, 1988; Michael & Seiler-Kelbitsch, 1972) and rice (Saha *et al.*, 1984; Saha *et al.*, 1986). In the present investigation, seed material of both cultivars in Experiment 2 were found to have very low levels of cytokinin-like activity when compared to the levels detected in the leaf material.

Zeatin, ribosylzeatin and glucosylzeatin have been tentatively identified in developing wheat grains (Jameson *et al.*, 1982), mature barley and wheat grains (Vizárová & Muzikova, 1981; Vizárová & Vozar, 1984) and mature rice grains (Saha *et al.*, 1984). In Experiment 1 of the present investigation, these compounds and a number of other cytokinins were tentatively identified in the seed material of the two wheat selections. Pooled data from HPLC separation of seed material in Experiment 2 indicate that although there was no signficant biological activity in either resistant or susceptible seed material, peaks of activity could be detected.

Cytokinin activity which co-chromatographed with zeatin, glucosylzeatin and ribosylzeatin was detected in the first leaf of barley and wheat cultivars (Vizárová, 1987; Vizárová *et al.*, 1986) and rice cultivars (Saha *et al.*, 1986). In the HPLC separation of leaf material in Experiment 2, the following cytokinins were tentatively identified in the line cultivar, zeatin-9-glucoside, zeatin-0-glucoside, *trans*-zeatin, dihydrozeatin, dihydroribosylzeatin and *iso*-pentenyladenine. Sephadex separation of resistant leaf material indicated the presence of *iso*-pentenyladenine, however, this compound could not be tentatively identified in the HPLC separation of this material. The susceptible cultivar was seen to have significant biological activity at retention times at which *iso*-pentenyladenine-9-glucoside and *iso*-pentenyladenosine markers eluted. The presence of *iso*-pentenyladenosine was also detected in the Sephadex separation of susceptible leaf material. As mentioned previously, Vizárová (1987) found that zeatin and its derivatives (at 100 μg per 3 cm^3) had an absolute inhibitory effect on the growth of *Erysiphe graminis*. Liu & Bushnell (1986) found no effects of zeatin (at concentrations of 10^{-6} - 10^{-4} M) on the development of this fungus on detached barley coleoptiles. The differences in concentrations, tissues and time of application could account for the different response observed by these two authors. The effect (if any) of the compounds identified in the resistant line of this study on the growth of *Puccinia graminis* f.sp. *tritici*, compared to the effect of those in the susceptible cultivar still needs to be determined.

Invasion of a resistant or susceptible host by a biotrophic plant pathogen ultimately results in an increase in the levels of endogenous cytokinins in both host types. However, the increase in the susceptible host has been found to be much greater than that in the resistant cultivar (Kern *et al.*, 1987; Sziraki *et al.*, 1976; Vizárová, 1974a, b, 1975b, 1979; Vizárová & Kováčová, 1980; Vizárová & Minarcic, 1974; Vizárová *et al.*, 1986). From the literature, the rôle of the pathogen in the changes of endogenous cytokinin levels detected, or the extent to which the pathogen contributes to the cytokinin pool of the infected plant, is unclear. Yadav & Mandahar (1981) were of the opinion that these increased levels reflect secretion of cytokinins by the pathogen, resulting in the formation of translocatory

sinks towards which nutrients would move. Dekhuijzen & Staples (1968) found that the cytokinin-like compounds detected in urediospores and mycelium of bean rust were not the same as those detected in infected plant tissue. Dekhuijzen (1976) thus concluded that infection stimulated the production of cytokinins by the host plant.

Qualitative changes in cytokinins have been observed after infection of barley with powdery mildew (Vizárová, 1973, 1974b, 1979, 1987) and wheat with stem rust (Vizárová *et al.*, 1986) and these authors supposed that the new compounds detected are produced by the pathogen in the host plants. What their results actually indicate is that the products of the pathogen do have an influence on the cytokinins detected in infected plants. However, it is unclear whether the new compounds detected are of pathogen origin or are the result of an influence of the products of the pathogen on the host metabolism of cytokinins. Evidence for the fact that the infection of plants by fungal pathogens might alter the quality of cytokinins detected in the infected plant material was presented by Mills & Van Staden (1978) and Nicholson & Van Staden (1988).

Vizárová and her co-workers (Vizárová, 1973, 1974a, b, 1975a, b, 1979, 1987; Vizárová & Kováčová, 1980; Vizárová & Minarcic, 1974) investigating the role of endogenous cytokinins in the barley- and wheat-powdery mildew interaction, have found that changes in cytokinin levels follow set patterns in resistant and susceptible plants. In both host types, there is an initial temporary decrease in cytokinin activity present in the leaf tissue while the pathogen becomes established, and a concomitant increase in cytokinin activity in the roots. The present author is of the opinion that at this stage of infection, in which there is the establishment of the biotrophic relationship between the host and the pathogen, there is active movement of metabolites into cells invaded by the fungus, possible enhanced by secretion by the fungus of cytokinin-like substances. The leaf, in order to re-establish the equilibrium of both cytokinin-like substances and metabolites, then a) moves cytokinins in from surrounding tissues, b) converts storage forms (glucosides) to active forms, c) induces the roots to biosynthesize more cytokinins. The temporary

rise recorded in the roots at this time (Vizárová, 1974b, 1975b, 1979; Vizárová & Minarcic, 1974) is an indication of increased biosynthesis or reduced export of cytokinins in the roots. Vizárová & Minarcic (1974) are of the opinion that, at 4dpi, the parasite actively inhibits the transport of cytokinins from the roots to the leaves, hence the increase in free cytokinins noted in roots at this time. In support of this opinion they cite Cole & Fernandes (1970) as having found that the cytokinins influence growth of the parasite in a negative way.

From an examination of the results of Vizárová (1975b, 1979) it would appear that the cytokinins in the roots move up into the above-ground parts of the plant, accumulating preferentially at the infection sites, hence the decrease in activity noted in the root tissue, and increase noted in the leaf tissue. The level in the root tissue of both resistant and susceptible cultivars drops back to the level in healthy controls while the levels in the leaves rise, reaching at peak at 6dpi in the resistant cultivar and rising continuously in the susceptible cultivar. It is during this period that spore formation is initiated in the susceptible cultivar, and as a result the fungus would require greatly enhanced levels of nutrients. With an increased cytokinin level there would be an increased mobilization of metabolites (Mothes & Engelbrecht, 1961; Mothes *et al.*, 1959;) to the infection sites. Vizárová (1975a) noted an enhanced production of cytokinin-like substances during the formation of fungal fructification organs in cultures of non-biotropic fungi. Possibly a similar increase in production by the powdery mildew fungus occurs in the susceptible host.

In the susceptible leaf tissue the activity is seen to rise through the period of spore formation and liberation (Vizárová, 1974b, 1975b, 1979), and this is reflected in the root as there is a great overall decrease in cytokinin activity measured in root tissue as the infection progresses (Vizárová, 1974b, 1975b, 1979; Vizárová & Minarcic, 1974). In the resistant leaf the activity is seen to rise temporarily (2 days) indicating that the cytokinins produced in the roots earlier are being transported to the infected leaves. The activity in the roots thus dropping back to the level seen in the roots of uninfected plants. After the rise seen at 6dpi there is then a decrease in activity measured in the leaf of resistant cultivars, to a level above that of the leaves of uninoculated plants (Vizárová, 1975b, 1979).

As there is no further increase detected in the roots of susceptible cultivars after the increase at 4dpi, yet the level in the leaf tissue rises (Vizárová, 1974b, 1975b, 1979; Vizárová & Minarcic, 1974), it is possible that in a susceptible reaction the fungus is able to produce cytokinins and these then move into the host leaf tissue and enhance the accumulation of metabolites and protein synthesis. Vizárová (1973, 1974b, 1979, 1987) is of the opinion that the appearance of a new zone of activity, corresponding to *iso*-pentenyladenine and its derivatives, in susceptible leaves could be an indication of cytokinin production by the fungus as this compound is not detected in susceptible cultivar roots, or in leaf and root material of resistant cultivars. Vizárová *et al.* (1986) detected the same zone of new activity in susceptible and moderately resistant wheat cultivars infected with *Puccinia graminis* f.sp. *tritici*, but not in fully resistant wheat cultivars. In the present investigation, in the absence of the pathogen, *iso*-pentenyladenine was tentatively identified in the resistant cultivar, and *iso*-pentenyladenine-9-glucoside in the susceptible line.

The maximum cytokinin activity measured at 6dpi in resistant cultivars (Vizárová, 1975b, 1979) indicates that the cytokinins produced in the roots (seen at 4dpi) have been transported to the infected leaves. The decrease seen at 6 - 10dpi indicates that once the cytokinins transported to the leaves become bound (used up ???), they are not replaced by movement of cytokinins from the fungus. The resistant reaction could thus come about as a result of the fungus, in the specific pathogen-host interaction, not being able to produce large amounts of cytokinins and thus failing to induce mobilization of metabolites preferentially into the cells with fungal haustoria. The degree of resistance of specific cultivars could depend on the cytokinin inducing/or producing capacities of the fungus once the biotrophic relationship has been established.

In the investigation of Vizárová (1979) the final level of cytokinin activity in the root, in both resistant and susceptible reactions, is lower than the initial activity in both healthy and inoculated plants. However, the activity in the final root reading (10dpi) for the susceptible cultivar is lower for the infected plants than the healthy controls, indicating a net removal of cytokinins from the roots of infected plants. In the

resistant cultivar, the final reading indicates that the root material of infected plants has a higher level than healthy control plants, thus indicating that removal of the cytokinins from the roots has been hampered, or stopped, or is not as intensive as that seen in the susceptible cultivar. This is a reflection of the situation in the leaf where in the resistant cultivar the "call for" cytokinins is not as great later in the interaction, while in the susceptible cultivar, excessive amounts of cytokinins are required for contribution to spore development and formation.

Hence, a measure of the differences in cytokinin activity between healthy and inoculated leaf material, at a time after inoculation with the pathogen would be sporulating in the susceptible cultivar, would possibly give an indication of the level of resistance that the cultivar has to the specific pathogen (taking into consideration the race and biotype, etc.). A resistant cultivar would have a much smaller overall increase in cytokinin activity than a susceptible cultivar. In the resistance interaction the fungus is thus unable either to produce greatly increased levels of cytokinins itself or to induce the biosynthesis of cytokinins by the host to any great extent. The genetics of the interaction would play a key controlling rôle here.

Thus, from the results of the present investigation, it can be concluded that leaf material of the wheat line Little Club *Sr25*, which is resistant to stem rust race 2SA4, does have a higher level of total cytokinin activity than that of the susceptible cultivar Little Club. Seed material of the resistant line did not always have higher levels of cytokinins than the susceptible cultivar. This is the first reporting of a comparison between cytokinin levels of isogenic wheat selections. A large number of cultivars would need to be tested before the usefulness of cytokinin levels as an indicator of resistance could be determined. The methodology used in this investigation would prove to be too labour-intensive and time-consuming for the plant breeder, and so if the cytokinin levels of wheat cultivars were found to be useful as an indicator of resistance in wheat breeding, more appropriate methods would need to be used.

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CHAPTER 2

SCANNING ELECTRON MICROSCOPY STUDY OF INFECTION STRUCTURE FORMATION BY *Puccinia graminis* f.sp. *tritici* ON AND IN THE UNIVERSAL SUSCEPTIBLE WHEAT CULTIVAR McNAIR

INTRODUCTION

Infection structure morphology of rust fungi pathogenic on grasses and cereals shows a considerable intertaxon variation and studies have indicated that infection structure morphology could provide additional distinctive traits to characterize rust species and subspecific taxa, especially independently from the host (Niks, 1986; Niks *et al.*, 1989)

Allen (1923) described her light microscope observations of infection structure development of *Puccinia graminis* f.sp. *tritici* Eriks. & Henn. in wheat. Today, her study remains remarkable for the detail it provides, the quality of which was rarely surpassed until the advent of electron microscopy. Scanning electron microscopy techniques especially are potentially valuable in the study of the morphology and ontogeny of infection structures. However, until recently, scanning electron microscopy studies of early infection structure formation of the rusts were limited to those describing structures differentiated from urediospores on artificial surfaces (Paliwal & Kim, 1974; Wynn, 1976; and Heath, M.C., (unpublished cited in Littlefield & Heath, 1979). The reason for this paucity of information is that leaf fracture methods of the type described by Gold *et al.* (1979), Mims (1981), and Beckett & Porter (1982) lend themselves to the examination of large fungal proliferations within host tissues rather than to the findings of early infection stages. Hughes & Rijkenberg (1985) described a leaf-fracturing technique which was adapted from the methods of Michelmore & Ingram (1981) and Al-Issa & Sigee

(1982). They used this technique to described the ontogeny and morphology of infection structures formed by *Puccinia sorghi* Schw. in the leaf of its uredial host, *Zea mays* L. Davies & Butler (1986) used a similar techique to describe the development of infection structures of the rust, *Puccinia porri* (Sow.) Wint., in leek (*Allium porrum* L.) leaves, as did Ferreira & Rijkenberg (1989) in describing development of *Uromyces transversalis* (Thum.) in gladiolus (*Gladiolus* L.) leaves.

The objective of the present study was to describe the formation and morphology of infection structures by *Puccinia graminis* f.sp. *tritici* in the universal stem-rust-susceptible wheat cultivar McNair employing the leaf fracturing technique of Hughes & Rijkenberg (1985).

MATERIALS AND METHODS

Rust propagation and inoculation. Freshly harvested urediospores of *Puccinia graminis* f.sp. *tritici* Eriks. & Henn. produced on 15-day-old susceptible wheat (*Triticum aestivum* L.) cv. Morocco plants in a greenhouse (18 - 30°C), were used to inoculate the first leaf of seven-day-old wheat cv. McNair at an inoculum dose of 50 mg urediospores per ml of Soltrol® 130 (Phillips Chemical Co.). A modified Andres & Wilcoxson (1984) inoculator was used to inoculate the seedlings. The Soltrol on the seedlings was allowed to evaporate for a hour and these were then placed in a dew chamber at 20°C and 100% relative humidity in the dark. A 12h/12h dark/light regime was followed. The inoculated leaves of ten seedlings were harvested at 6, 12, 24, 48, 96 and 144 hours-post-inoculation (hpi). At 24 hpi the remaining seedlings were removed from the dew chamber and placed on a bench in a greenhouse at a maximum temperature of 24°C.

Specimen preparation. The harvested leaf pieces were fixed in 3% glutaraldehyde in a 0.05 M sodium cacodylate buffer, pH 6.82 - 7.24, for 8 h or overnight, washed twice in buffer, post-fixed for 2 hours in 2% osmium tetroxide in buffer, washed

twice in buffer, and dehydrated in a graded ethanol series. The material was then critical point dried in carbon dioxide after a graded transition from ethanol to amyl acetate. The leaf pieces were cut into 3 x 3 mm squares and mounted on stubs. The leaf fracture technique of Hughes & Rijkenberg (1985) was performed on material harvested at 12 - 96 hpi. Leaf pieces harvested at 6 and 144 hpi were left unfractured. All specimens were gold/palladium-coated in a Polaron® Sputter coater and examined using a Hitachi® S-570 scanning electron microscope operating at 5 or 8 kV. Since infection structures remain attached to the epidermis, only the stripped epidermis of fractured material was examined, whereas inoculated leaf surfaces of material harvested at 6 and 144 hpi were scanned.

OBSERVATIONS

The leaves of wheat are parallel-veined and have stomata regularly arranged in longitudinal rows along their length. The long axes of the stomata are orientated parallel to the long axis of the leaf.

At germination, a germ tube is extruded through a germ pore in the urediospore wall and ramifies over the leaf surface, generally perpendicularly to the long axis of the leaf (Plate 1 Fig. a). Short exploratory branches are formed at the anticlinal walls of any epidermal cell encountered (Plate 1 Fig. a). Once a stoma is located, a terminal appressorium is formed (Plate 1 Fig. b). Appressoria were observed at 6 hpi. Collapsed appressoria remain adherent to the leaf surface even after 144 hpi. In a number of cases two appressoria were seen over a single stoma.

The lower appressorial surface has a rugose texture. A ridge that follows the external contours of the stomatal slit develops on the lower appressorial surface, and from this ridge, a single infection peg arises and penetrates at one end of the stomatal slit. In the substomatal chamber, the infection peg swells into a substomatal vesicle (SSV) initial (Plate 1 Fig. c), the blade-shaped connection (see remnant viewed from above in Plate 1 Fig. c and from the side in Plate 1 Fig. d)

between the appressorium and the SSV having been termed the interconnective tube by Hughes & Rijkenberg (1985). From a size analysis of 30 SSVs at 12 hpi, it appears that, on emergence from the stomatal slit, the SSV initial elongates parallel to the stomatal slit, the smallest SSV observed measuring 6.8 by 3.6 μm. The SSV initial progressively swells to a more rounded shape (approximately 8.6 by 6.4 μm) and then increases in size in both dimensions until it attains a size of approximately 12 by 8 μm. Further increase in length is associated with a slight increase in breadth, the most mature SSVs at 12 hpi measuring 15 - 17 by 6 - 7 μm. A sample of material harvested at 6 hpi which was inadvertently fractured revealed a few ovoid SSVs indicating that SSV development closely follows appressorium formation.

The SSVs were orientated such that their long axes were parallel to the long axis of the stomatal chamber and hence the long axis of the leaf (Plate 2 Figs. a - c). Near-spherical SSVs observed at later sampling times, as well as collapsed SSVs, were considered to be aborted structures. A number of stomata were seen on which two SSVs had developed (Plate 3 Fig. a). The SSVs then elongate unilaterally, closely appressed to the inner epidermal surface, in a direction parallel to the long axis of the leaf, to form a primary infection hypha (Plate 3 Fig. c). Primary infection hyphae are approximately 4 μm wide and are thus narrower than SSVs. Where a primary infection hypha abuts on to a host cell, a septum forms, delimiting a haustorial mother cell (HMC) at the tip (Plate 4 Fig. a). Primary HMCs were commonly seen to abutt onto the epidermal cell adjacent to the swollen end of a stomatal guard cell (Plate 4 Figs. a-d), and were never seen to form in association with the subsidiary cell. By 12 hpi, although primary infection hyphae had been formed at some infection sites, at the majority of sites early stages of SSV development were found. Small numbers of collapsed SSVs were also observed at 12 hpi (Table 1). Relatively more collapsed SSV initials were recorded at 48 and 96 hpi although numbers were low (Table 1). Some SSVs (often spherical) produced primary infection hyphae that were very elongated, and which showed no septum formation (Plate 3 Fig. b). Since none of such structures was seen to have developed beyond this stage, these were regarded as abortive. Low numbers of

these atypical primary infection hyphae were observed at 48 hpi and at all subsequent sampling times (Table 1).

Table 1 Counts* (%) of infection structures of *Puccinia graminis* f.sp. *tritici* that had developed to the indicated levels on wheat at specific time intervals post-inoculation

INFECTION STRUCTURE	Hours-post-inoculation (hpi)**			
	12	24	48	96
Normal Development				
Ovoid substomatal vesicle	83	89	80	73
Primary infection hypha	25	79	45	55
Primary infection hypha with haustorial mother cell	17	69	43	54
Secondary infection hypha	-	41	36	50
Intercellular mycelium and haustorial mother cell	-	2	24	41
Abnormal Development				
Collapsed ovoid substomatal vesicle	7	1	2	5
Spherical substomatal vesicle	9	9	6	6
Collapsed spherical substomatal vesicle	1	2	9	16
Atypical primary infection hypha	-	-	3	1
Total number of sites	102	123	181	303

* Percentages based on cumulative totals

** Observations were also made at 144 hpi for wheat and data for this time followed the pattern shown in this table

See Appendix 2.1 for real counts

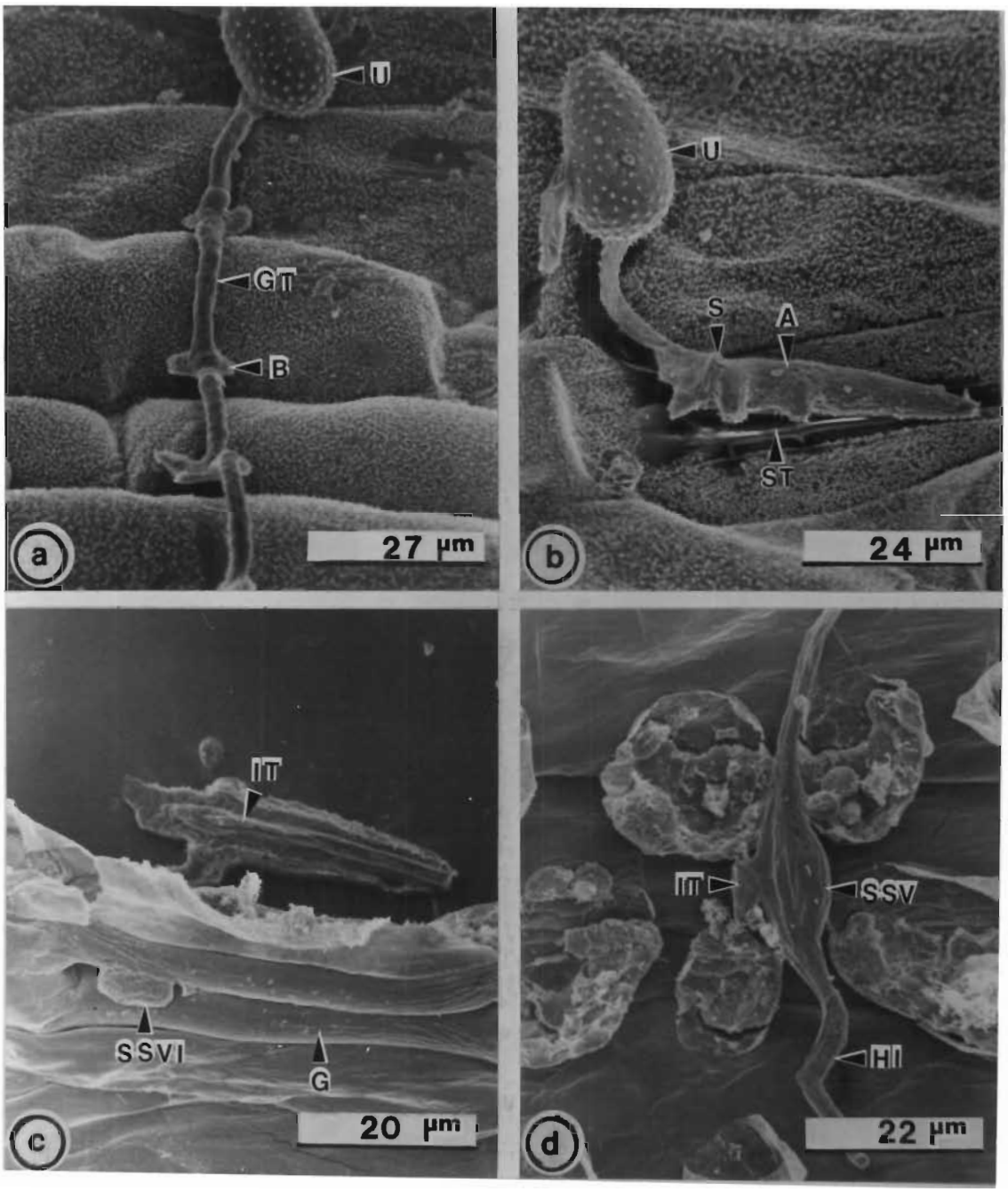

U
GT
B
a
27 µm
U
S
A
ST
b
24 µm
IT
SSVI
G
c
20 µm
IT
SSV
HI
d
22 µm

PLATE 2

Development of *Puccinia graminis* f.sp. *tritici* on and in the susceptible wheat cv. McNair

(a) Near-spherical substomatal vesicle initial in wheat at 12 hpi

(b) Elongate substomatal vesicle in wheat at 12 hpi

(c) Mature substomatal vesicle in wheat at 24 hpi

Abbreviations:

SSVI = Substomatal vesicle initial
SSV = Substomatal vesicle
G = Guard cell

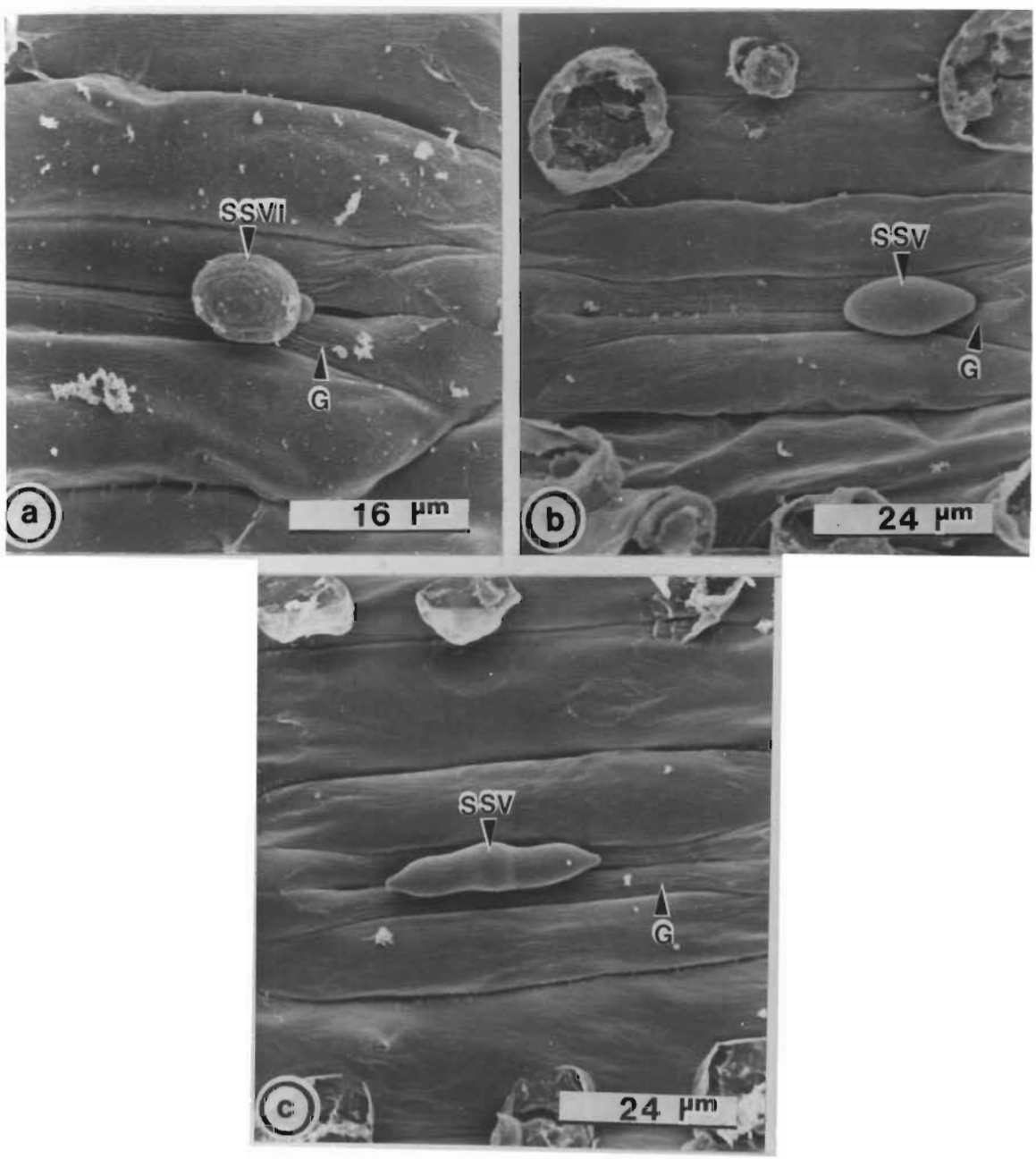

SSVI
G
a
16 µm
SSV
G
b
24 µm
SSV
G
c
24 µm

PLATE 3

Development of *Puccinia graminis* f.sp. *tritici* on and in the susceptible wheat cv. McNair

(a) Two substomatal vesicles beneath wheat stoma at 48 hpi

(b) Abnormal primary infection hypha in wheat at 48 hpi

(c) Primary infection hypha without septum in wheat at 12 hpi

Abbreviations:

SSV = Substomatal vesicle
G = Guard cell
HI = Primary infection hypha

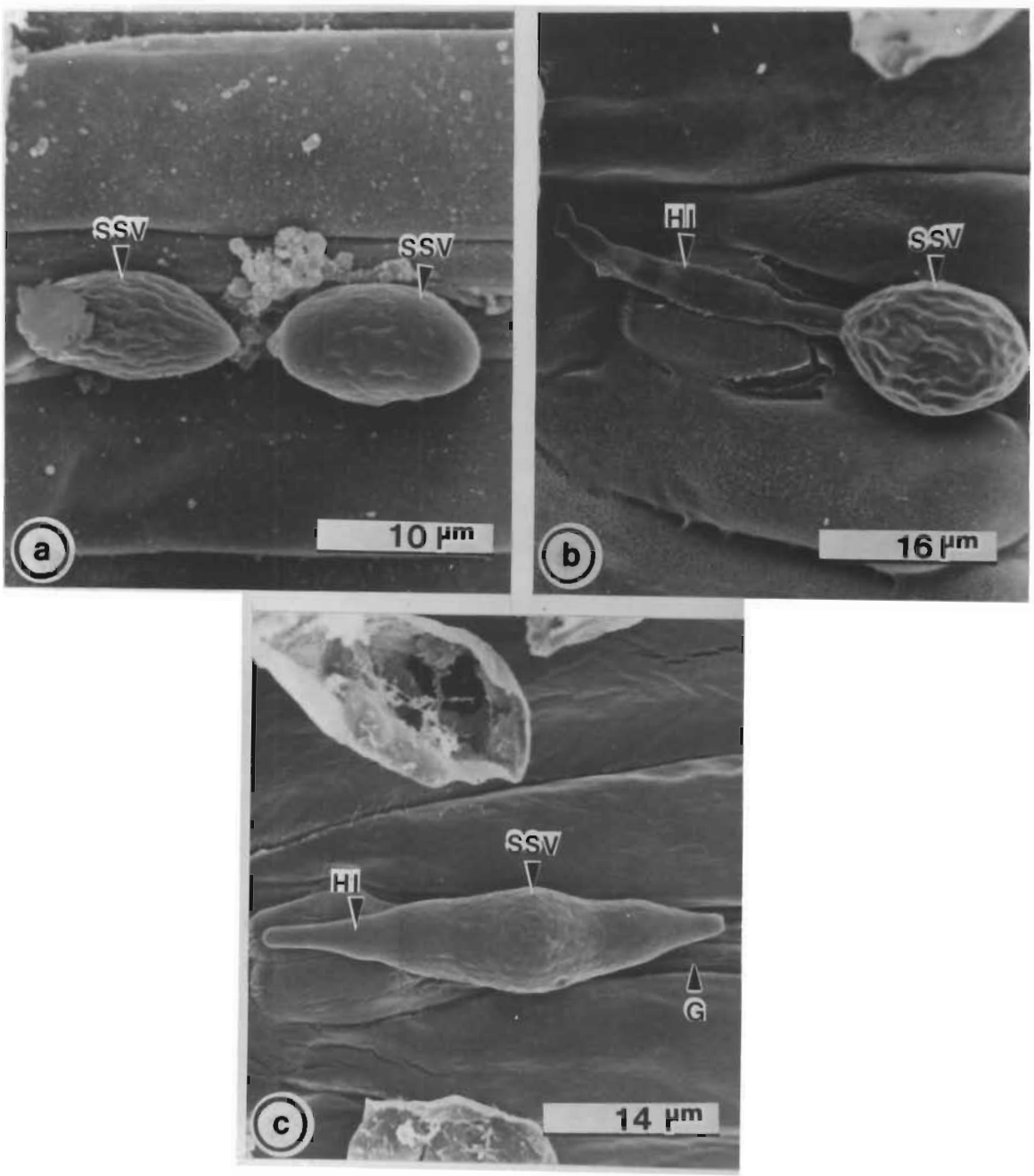

SSV
SSV
10 µm
a
HI
SSV
16 µm
b
SSV
HI
G
14 µm
c

PLATE 4

Development of *Puccinia graminis* f.sp. *tritici* on and in the susceptible wheat cv. McNair

(a) Primary infection hypha with haustorial mother cell in wheat at 12 hpi

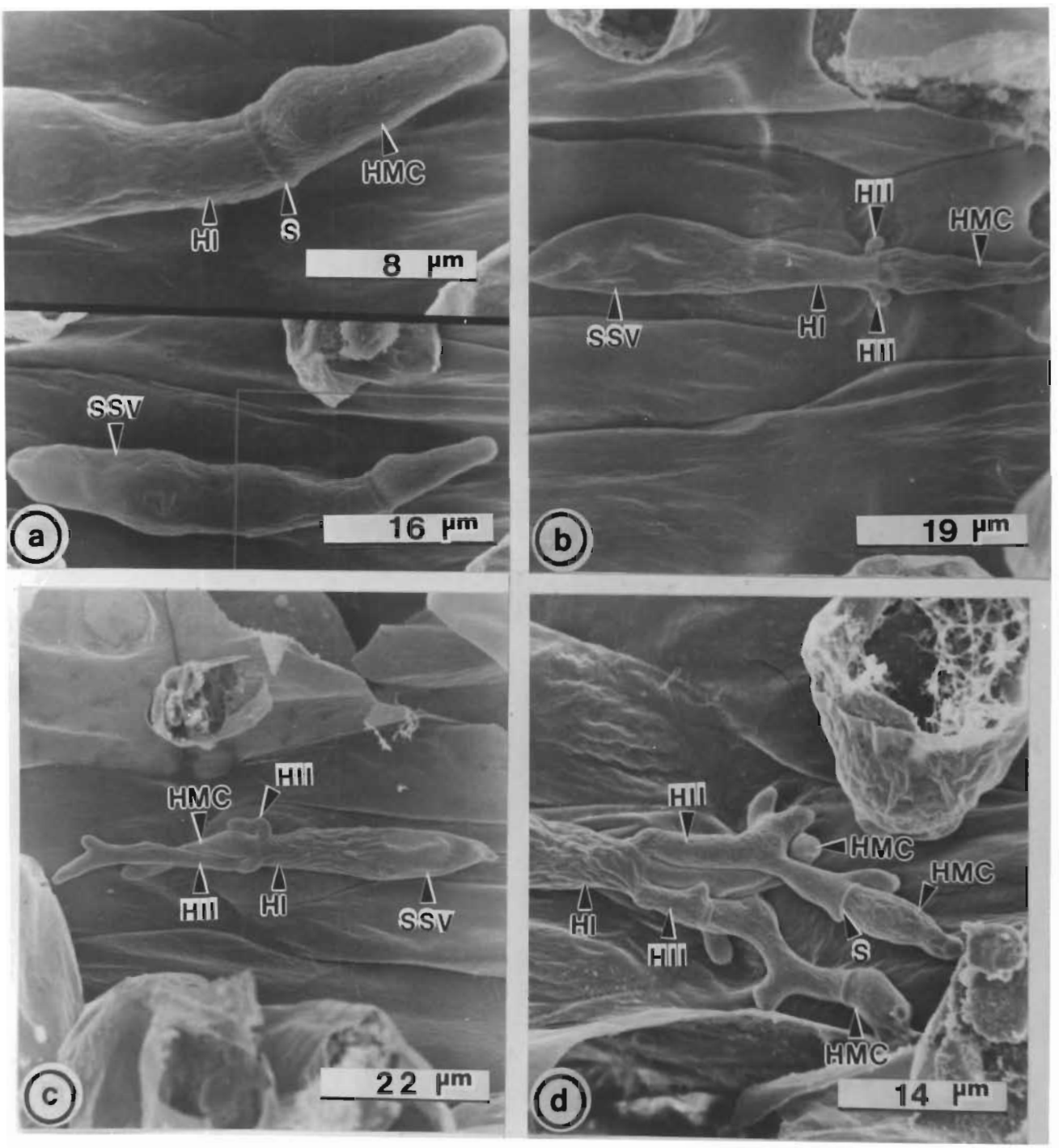
(b) Secondary infection hypha initials in wheat, arising on the substomatal vesicle side of the haustorial mother cell septum (24 hpi)

(c) Elongated secondary infection hyphae in wheat at 24 hpi

(d) Haustorial mother cells arising from secondary infection hyphae in wheat at 48 hpi

Abbreviations:

S = Septum
HI = Primary infection hypha
SSV = Substomatal vesicle
HII = Secondary infection hypha
HMC = Haustorial mother cell


HMC
HI
S
8 µm
SSV
16 µm
a
HII
HMC
SSV
HI
HII
19 µm
b
HMC
HII
HII
HI
SSV
22 µm
c
HII
HMC
HMC
HI
HIII
S
HMC
14 µm
d

Once the SSV and primary infection hypha have expanded fully, and the first HMC has been delimited on the primary hypha, secondary infection hyphae emerge at a position on the SSV side of the septum separating the HMC from the primary infection hyphae (Plate 4 Fig. b). Generally two secondary infection hyphae emerge at the septum, though three and four have been observed. By 24 hpi there were secondary infection hyphae at most infection sites (Table 1). Secondary infection hyphae elongate (Plate 4 Fig. c), and, by septum formation, cut off a terminal HMC (Plate 4 Fig. d). HMCs were first observed on secondary infection hyphae at 24 hpi in two out of 123 sites examined (Table 1). By 48 hpi many secondary hyphae had formed HMCs (Table 1). HMCs are generally larger in diameter than primary or secondary infection hyphae, and intercellular hyphae. Further branching occurs on the proximal side of the HMC septum. Secondary infection hyphae with HMCs give rise to the intercellular hyphae and in this manner the fungal thallus develops.

At 144 hpi, uredia with a number of immature urediospores were observed.

DISCUSSION

Littlefield & Heath (1979) have reviewed the literature on infection structure formation. The general sequence of infection structure formation and development of *Puccinia graminis* f.sp. *tritici* on the susceptible wheat cv. McNair closely follows that described by Allen (1923) for *Puccinia graminis* f.sp. *tritici* on the susceptible wheat cv. Baart.

The germ tubes of *Puccinia graminis* f.sp. *tritici* were seen to extend perpendicularly to the long axis of the leaf. Johnson (1934) first noted that urediospore germ tubes of *Puccinia graminis* f.sp. *tritici* grow predominantly along the transverse axis of the plant leaf. He postulated that the directional growth may be a thigmotropic response to the plant surface. Lewis & Day (1972) proposed that, as the epicuticular wax layer is the only leaf part in direct contact with the germ tube, this must be the structure to which the germ tube responds.

Wynn (1976) demonstrated that the germ tubes of *Uromyces phaseoli* var. *typica* grow at right angles to the large ridges formed by the curvature of the host epidermis cells. However, Hughes & Rijkenberg (1985) recorded that *Puccinia sorghi* germ tubes grow towards maize stomata randomly as they traverse both axes of the leaf surface, not only by extending across epidermal cells, but also by following the depressions along both the short and the long anticlinal walls of epidermal cells.

Allen (1923) observed a septum separating the germ tube and appressorium of *Puccinia graminis* f.sp. *tritici*. This septum was observed in the present SEM study on *Puccinia graminis* f.sp. *tritici*.

The blade-like infection peg which the appressorium pushes through the stomatal slit prior to SSV initial formation was first described for *Puccinia graminis* f.sp. *tritici* by Allen (1923). Hughes & Rijkenberg (1985) presented evidence that the infection peg of *Puccinia sorghi* may penetrate the stomatal slit first at both ends of the stoma, then centripetally. In the present study, the infection peg was observed to arise unilaterally from the appressorial ridge, progressive intrusion lining the stomatal slit, giving rise to the blade-like wedge. The septum between SSV and interconnective tube observed by Hughes & Rijkenberg (1985) and Davies & Butler (1986) could not be demonstrated unequivocally for *Puccinia graminis* f.sp. *tritici* as the SEM technique is not always capable of resolving septa.

The SSV initial balloons out near-spherically in the substomatal chamber before assuming an ovoid shape prior to formation of the primary hypha. The considerable number of spherical and ovoid SSVs, in both collapsed and non-collapsed state, persisting at later sampling times, indicates their inability to establish normal host-pathogen interactions, and supports the contention of Hughes & Rijkenberg (1985) that uredial propagules are not equal in inherent aggressiveness, or that some form of host resistance is expressed even in the susceptible host. The relatively high numbers of SSV initials and SSVs, which failed to develop further, observed at the later harvesting times (48 and 96 hpi) might also indicate that some urediospores

take much longer to germinate and infect the host. Niks (1990) found that within leaves of barley (*Hordeum vulgare* L) great variation in fate among individual sporelings of *Puccinia hordei* Otth., and a negative association was evident between germ tube length of sporelings and i) the chance of successful colony establishment, and ii) the size of the established colony. This author suggested that the formation of a long germ tube decreases the amount of energy available to the sporeling to infect the host. It is probable that variation in germ tube lengths of urediospores in part contributes to the variation in fate of the propagules in the present investigation. It appears that approximately 50% of all infections following successful penetration have aborted by 96 hpi.

The observation, in this study, of more than one apparently functional substomatal vesicle occupying the same stomatal chamber, has previously been recorded in a number of host-rust interactions (Allen, 1923; Davies & Butler, 1986; Ferreira & Rijkenberg, 1989; Hughes & Rijkenberg, 1985).

Transmission electron microscopy will be required to confirm whether, unlike the two-celled primary infection hyphae of *Puccinia sorghi* (Hughes & Rijkenberg, 1985) and *Puccinia porri* (Davies & Butler, 1986), those of *Puccinia graminis* f.sp. *tritici* are single celled. The terminal cell of the primary hypha of *Puccinia sorghi* (Hughes & Rijkenberg, 1985) and *Puccinia porri* (Davies & Butler, 1986) was observed to be a haustorium mother cell. A fluorescence microscopy investigation (Lennox & Rijkenberg, 1989) demonstrated the presence of a haustorium within the host epidermal cell onto which the terminal cell of a primary hypha abutted. Thus it can be concluded that these terminal cells are haustorial mother cells. Hughes & Rijkenberg (1985) suggested that secondary hypha formation, and subsequent development of the vegetative mycelium, is dependent on the prior establishment of successful host-pathogen relations by the primary hypha via the formation of a haustorium.

In *Puccinia sorghi* (Hughes & Rijkenberg, 1985), and *Puccinia graminis* f.sp. *tritici*, secondary infection hyphae arise on the SSV side of the septum. Such

secondary hyphae delimit HMCs and further proliferate into the intercellular mycelium.

A comparison of the developmental time frame recorded for *Puccinia sorghi* by Hughes & Rijkenberg (1985) with that of *Puccinia graminis* f.sp. *tritici* in the present study, reveals that these two rusts are very similar in the time required to reach a particular infection stage. Both rusts have formed at least some SSV initials by 6 hpi and primary infection hyphae by 12 to 14 hpi. By 48 hpi, both rusts have formed secondary infection hyphae, and haustorium mother cells have been delimited from such hyphae.

The leaf-fracturing technique described by Hughes & Rijkenberg (1985) is simple to perform on leaf tissues that fracture easily, as wheat did, and is particularly useful for studies on the infection structure morphology of rust fungi which initially proliferate in a horizontal, rather than a vertical manner in the leaf. The present investigation has provided a clear picture of infection structure formation and morphology of *Puccinia graminis* f.sp. *tritici* in wheat.

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CHAPTER 3

SCANNING ELECTRON MICROSCOPY STUDY OF INFECTION STRUCTURE FORMATION BY *Puccinia graminis* f.sp. *tritici* ON AND IN THREE CEREAL SPECIES

INTRODUCTION

The infection of higher plants by organisms, normally pathogenic on other species or genera, results in the expression of the most common, and the most effective forms of naturally occurring disease resistance (Heath, 1977). Non-host plants of of pathogens are a potential source of resistance genes for host plants of a pathogen (Niks, 1987) and a study of the resistance mechanisms expressed by the non-hosts could provide valuable information in the selection of new resistant host plants. The elucidation of the mechanisms of non-host resistance has been the subject of a number of investigations and review articles (Fernandez & Heath, 1985; Heath, 1972; Heath, 1974; Heath, 1977; Heath, 1981; Heath, 1982; Heath, 1983; Heath & Stumpf, 1986; Leath & Rowell, 1966; Leath & Rowell, 1969; Leath & Rowell, 1970; Niks, 1983; Niks & Dekens, 1987; Sellam & Wilcoxson, 1976; Stumpf & Heath, 1985; Wood & Heath, 1986). All of these studies have involved the use of light or transmission electron microscopy techniques. Hughes & Rijkenberg (1985) published a leaf-fracturing technique which facilitates the observation of within-leaf infection structures. This technique has been used in a preliminary study describing the development of *Puccinia graminis* f.sp. *tritici* in a susceptible and a resistant wheat cultivar (Lennox & Rijkenberg, 1985) and a similar technique was used by Ferreira & Rijkenberg (1989) to describe development of infection structures of *Uromyces transversalis* (Thum.) Winter in leaves of the host (*Gladiolus* L.) and a non-host (*Zea mays* L.). The objectives of the present study were to describe and compare the development of *Puccinia graminis* f.sp. *tritici* infection structures in non-host plant species

and to compare their development with those on the susceptible wheat cultivar McNair as described in Chapter 2.

MATERIALS AND METHODS

Rust propagation and inoculation. Freshly harvested urediospores of *Puccinia graminis* f.sp. *tritici* Eriks. & Henn., produced on 15-day-old susceptible wheat (*Triticum aestivum* L.) cv. Morocco plants in a greenhouse (18 - 35°C), were used to inoculate the adaxial surfaces of the third leaf of 7-day-old sorghum (*Sorghum caffrorum* L.) cultivar PNR 8469, the second leaf of 7-day-old barley (*Hordeum vulgare* L.) cultivar Diamant and the third leaf of 15-day-old maize (*Zea mays* L.) cultivar B14rp, at an inoculum dose of 50 mg urediospores per ml of Soltrol® 130 (Phillips Chemical Co.). An investigation of *Puccina graminis* f.sp. *tritici* development on and in the susceptible wheat cultivar McNair (Chapter 2) was conducted simultaneously with the present investigation, the results of which were used for comparative purposes in the present study. A modified Andres & Wilcoxson (1984) inoculator was used to inoculate the plants. The seedlings were allowed to dry for an hour and were then placed in a dew chamber at 20°C and 100% RH in the dark. A 12h/12h dark/light regime was followed. The inoculated leaves of ten seedlings of each plant type were harvested at 6, 12, 24, 48 and 96 hours-post-inoculation (hpi). At 24 hpi the remaining seedlings were removed from the dew chamber and placed on a bench in a greenhouse at a maximum temperature of 24°C.

Specimen preparation. The same specimen preparation methods as those described in Chapter 2 were followed in this study. In addition to the observations on infection structures within the leaf at 6, 12, 24, 48, and 96 hpi, the outer epidermal surfaces of non-host material sampled at six hpi were also examined.

OBSERVATIONS

The morphology of infection structures and the pattern of infection structure

development on and in barley, sorghum and maize were found to resemble closely those on the susceptible wheat host McNair (Chapter 2). On all of these species, germ tube growth was perpendicular to the long axis of the leaf, and by six hpi, urediospores had germinated, appressoria had formed and, after penetration, substomatal vesicle (SSV) development had commenced (Plate 1 Figs. a, b and c for sorghum; Plate 2 Figs. a, b and c for maize; Plate 4 Figs. a, b and c for barley). Mature SSV long axis orientation in all four cereal species was parallel to the long axis of the stomatal opening, and hence the long axes of the leaves (Plate 1 Figs. c and d; Plate 2 Fig. c; Plate 4 Fig. c). By 12 hpi, infection in sorghum had progressed to the primary infection hypha stage without the presence of a haustorial mother cell (HMC) (Plate 1 Fig. d). Infection on sorghum was never seen to have developed beyond this stage (Table 1). In maize at 12 hpi however, infection showing a primary infection hypha with a septum delimiting a HMC was observed (Plate 3 Fig. a) (Table 1). Infection in barley at 12 hpi had progressed to primary infection hyphae with HMCs, secondary infection hyphae and further hyphal proliferation with HMCs at many of the sites (Plate 4 Fig. d, Plate 1 Figs. a, b).

At 24 hpi, infection in maize had progressed to the formation of secondary infection hyphae, and samples taken thereafter revealed that infection in maize did not progress beyond that shown in Plate 3 Fig. b. In barley at 24 hpi and all later sampling times, HMCs were observed on secondary infection hyphae (Plate 1 Figs. c,d)(Table 1).

In all three species, as well as in wheat, some collapsed SSVs, as well as atypical SSVs and primary infection hyphae were observed (Table 1).

Counts of infection structures of *Puccinia graminis* f.sp. *tritici* observed on maize, sorghum and barley at six, 12, 24, 48 and 96 hpi are recorded in Table 1. See also Appendix 3.1.

Table 1 Counts* (%) of infection structures of *Puccinia graminis* f.sp. *tritici* that had development to the indicated levels on maize, sorghum and barley at specific time intervals post-inoculation

INFECTION STRUCTURE	HOURS-POST-INOCULATION (hpi)											
	SORGHUM				MAIZE				BARLEY			
	12	24	48	96	12	24	48	96	12	24	48	96
Normal Development												
Ovoid substomatal vesicle	65	59	100	67	91	77	73	86	76	90	62	89
Primary infection hypha	27	3	30	23	38	67	62	73	59	68	40	69
Primary infection hypha with haustorial mother cell					10	11	12	18	56	55	31	62
Secondary infection hypha						1	4	18	47	30	5	56
Intercellular mycelium with haustorial mother cells									31	4	1	53
Abnormal Development												
Collapsed ovoid substomatal vesicle				10			2		2	1	13	2
Spherical sub-stomatal vesicle	35	24		10	8	4	6	9	6	7	3	2
Collapsed spherical substomatal vesicle		9			1		4		14	1	11	5
Atypical primary infection hypha		8		7		19	14	5	1	2	10	2
Number of sites observed	37	75	47	30	153	73	94	22	236	212	220	55

* Percentage based on cumulative totals

See Appendix 3.1 for real counts

PLATE 1

Development of *Puccinia graminis* f.sp. *tritici* on and in the sorghum cv. PNR 8469

(a) Germ tube and appressorium on sorghum at 6 hpi

(b) Substomatal vesicle initial in sorghum at 6 hpi

(c) Substomatal vesicle in sorghum at 6 hpi

(d) Primary infection hypha without septum, at 12 hpi in sorghum

Abbreviations:

U = Urediospore
GT = Germ tube
A = Appressorium
SSVI = Substomatal vesicle initial
SSV = Substomatal vesicle
G = Guard cell
HI = Primary infection hypha

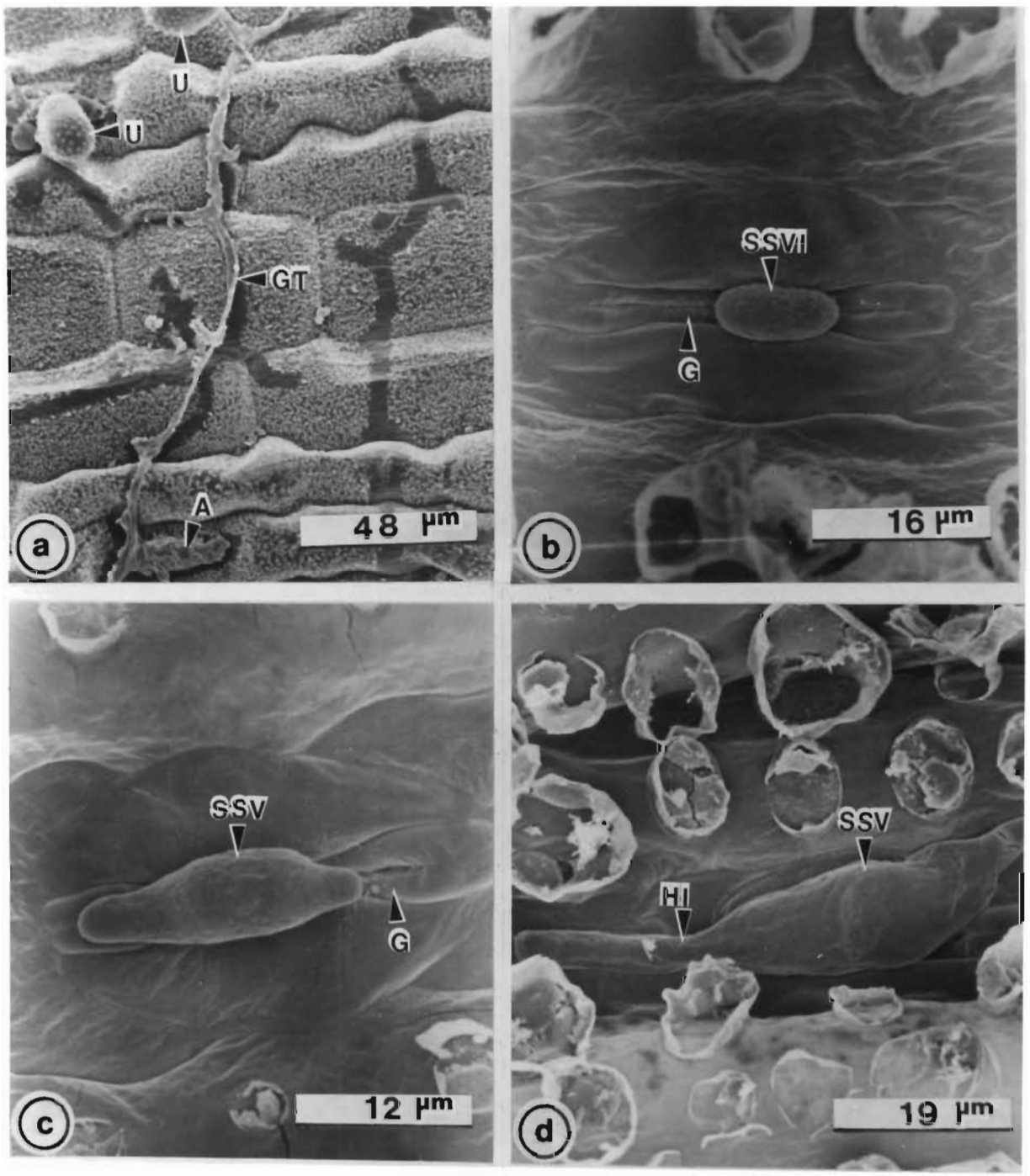

U
U
GT
A
48 µm
a
SSVI
G
16 µm
b
SSV
G
12 µm
c
SSV
HI
19 µm
d

PLATE 2

Development of *Puccinia graminis* f.sp. *tritici* on and in the maize cv. B14rp

(a) Germ tube growing perpendicular to the long axis of the maize leaf, and terminating in an appressorium at 6 hpi

(b) Germ tube and appressorium on maize at 6 hpi

(c) Substomatal vesicle in substomatal chamber of maize at 6 hpi

Abbreviations:

A = Appressorium
GT = Germ tube
U = Urediospore
G = Guard cell
SSV = Substomatal vesicle

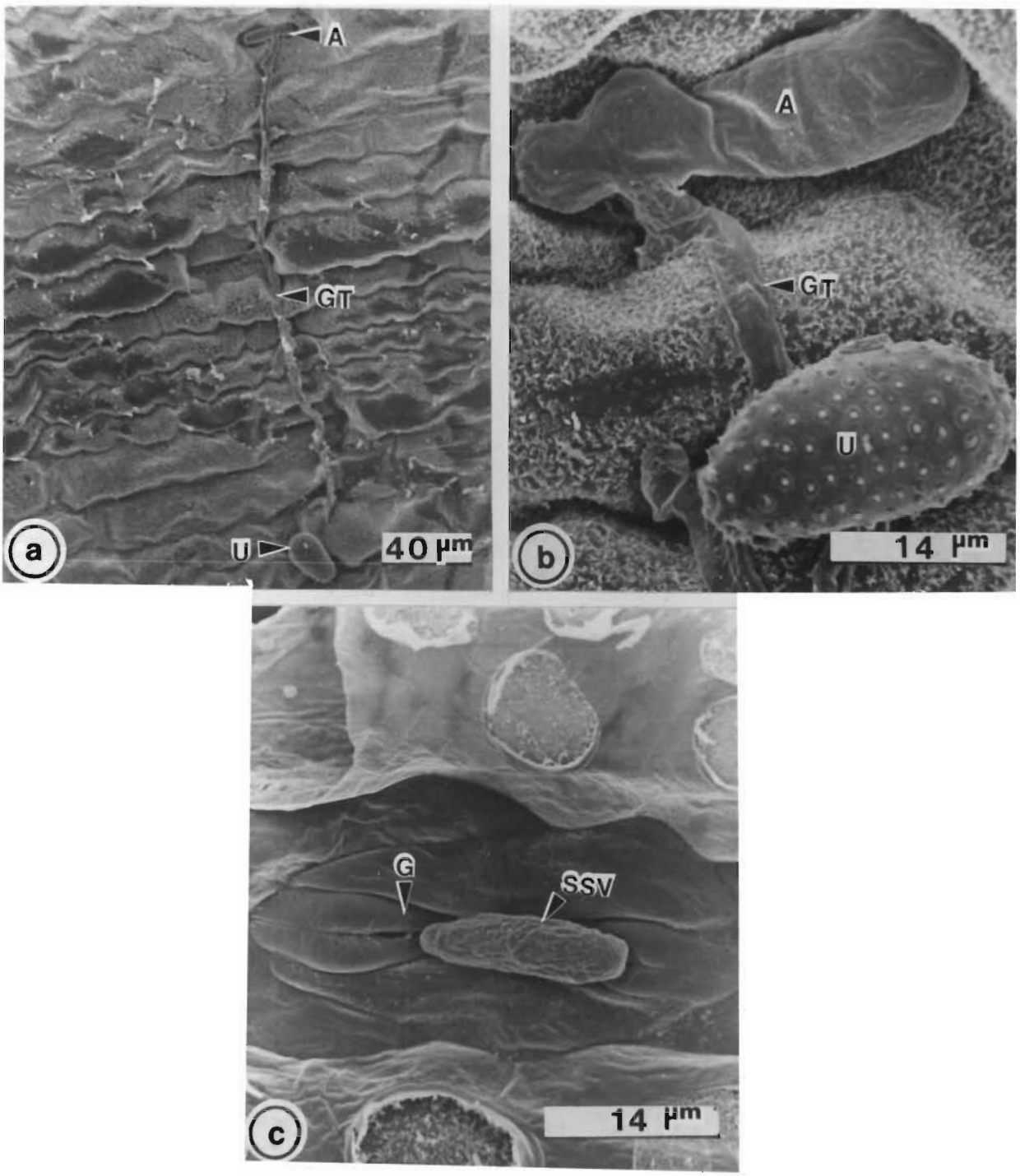

A
GT
U
40 µm
a
A
GT
U
14 µm
b
G
SSV
14 µm
c

PLATE 3

Development of *Puccinia graminis* f.sp. *tritici* on and in the maize cv. B14rp

(a) Primary infection hypha with septum delimiting a haustorial mother cell, at 12 hpi in maize

(b) Secondary infection hyphae in maize at 24 hpi

Abbreviations:

SSV = Substomatal vesicle
S = Septum
HI = Primary infection hypha
HII = Secondary infection hypha
HMC = Haustorial mother cell

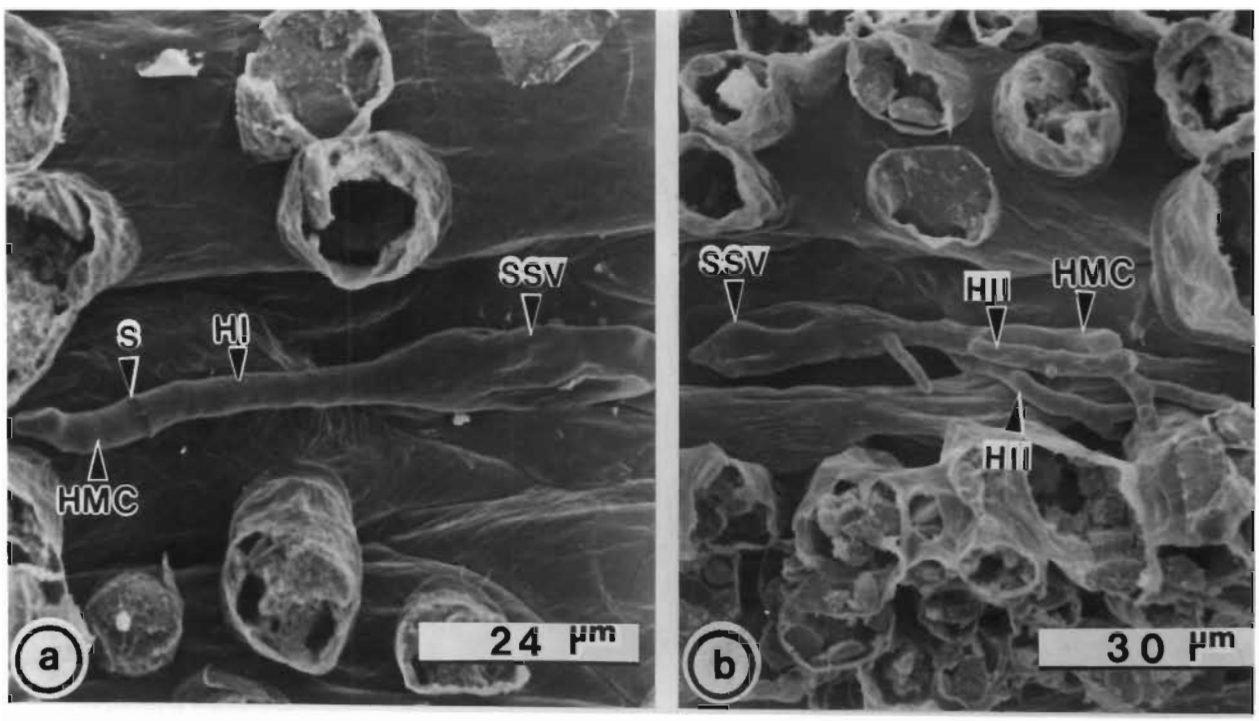

SSV
S
HI
HMC
24 µm
a
SSV
HII
HMC
HII
30 µm
b

PLATE 4

Development of *Puccinia graminis* f.sp. *tritici* on and in the barley cv. Diamant

(a) Germ tube and appressorium on barley at 6 hpi

(b) Substomatal vesicle initial in barley at 6 hpi

(c) Substomatal vesicle in barley at 6 hpi

(d) Primary infection hypha with haustorial mother cell, at 12 hpi in barley

Abbreviations:

U = Urediospore
GT = Germ tube
A = Appressorium
SSVI = Substomatal vesicle initial
SSV = Substomatal vesicle
G = Guard cell
HI = Primary infection hypha
S = Septum
HMC = Haustorial mother cell

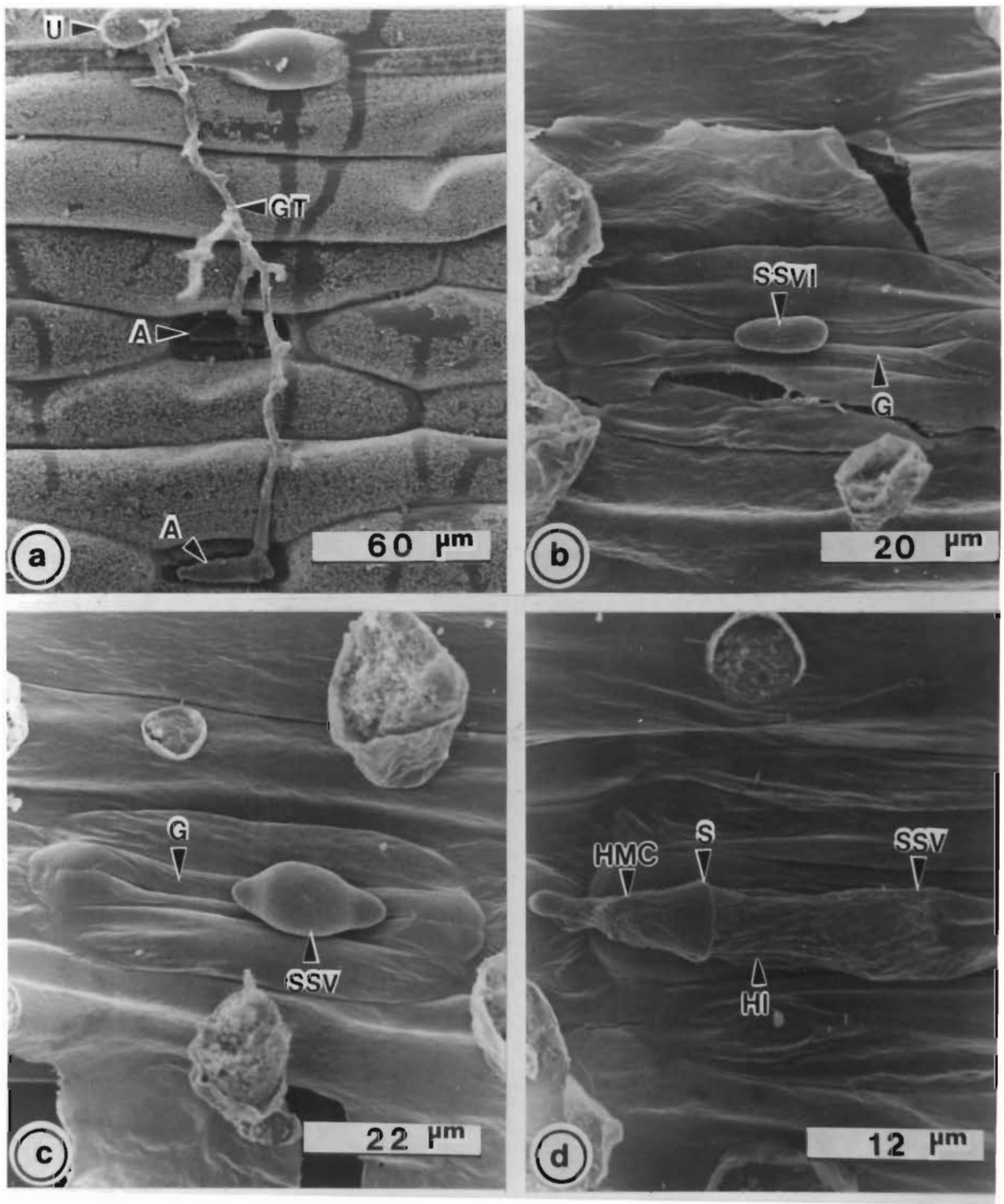

U
GT
A
A
60 µm
a
SSVI
G
20 µm
b
G
SSV
22 µm
c
HMC
S
SSV
HI
12 µm
d

PLATE 5

Development of *Puccinia graminis* f.sp. *tritici* on and in the barley cv. Diamant

(a) Secondary infection hyphae in barley at 12 hpi

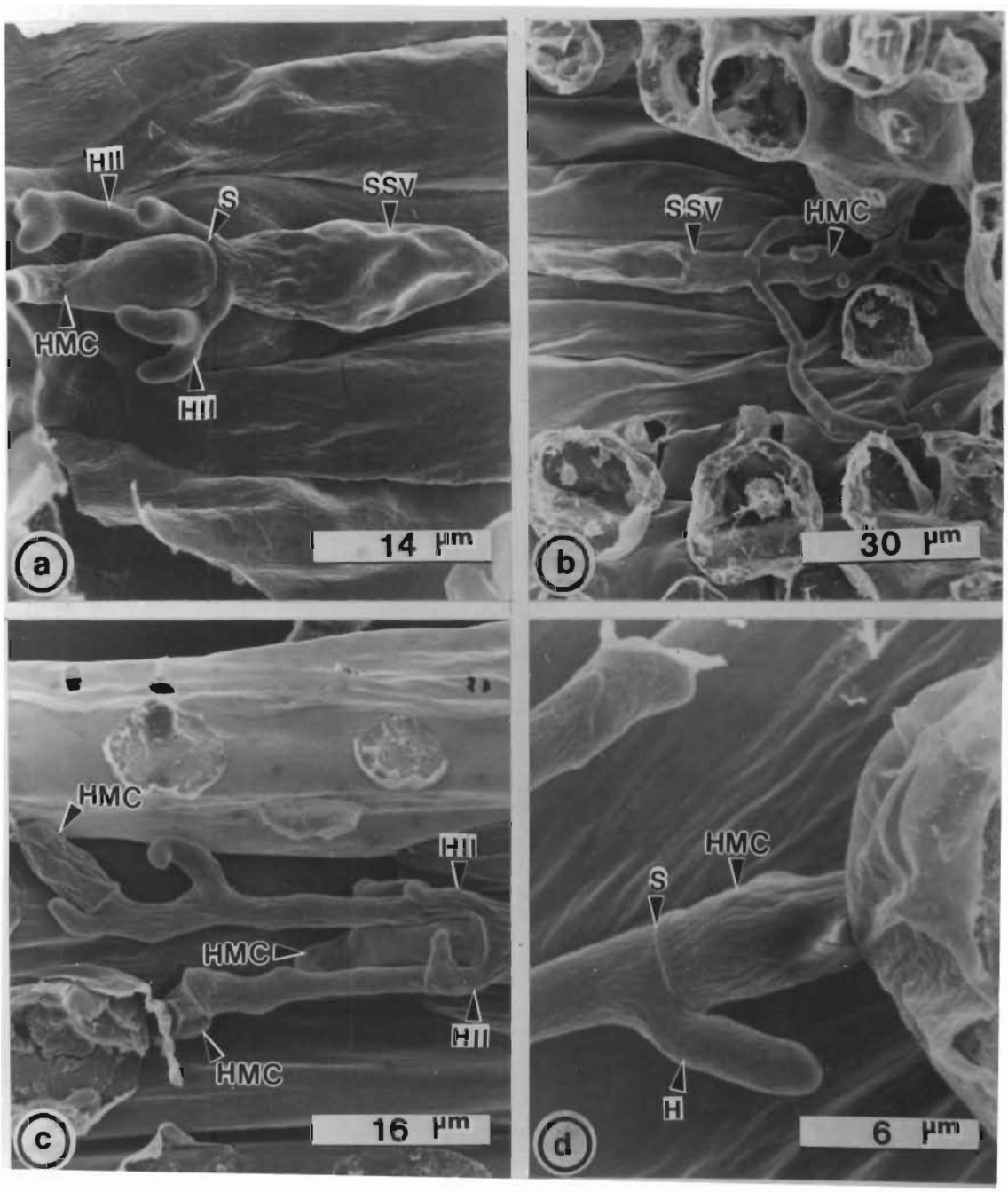
(b) Thallus formation in barley at 12 hpi

(c) Haustorial mother cells at tips of secondar infection hyphae in barley at 24 hpi

(d) Hyphal branch arising from behind the haustorial mother cell septum of a secondary infection hypha in barley at 24 hpi

Abbreviations:

SSV = Substomatal vesicle
HI = Primary infection hypha
HII = Secondary infection hypha
S = Septum
HMC = Haustorial mother cell
H = Hyphal branch


HII
S
SSV
HMC
HII
14 µm
a
SSV
HMC
30 µm
b
HMC
HII
HMC
HII
HMC
16 µm
c
HMC
S
H
6 µm
d

DISCUSSION

The morphology and general pattern of initial development of *Puccinia graminis* f.sp. *tritici* infection structures on sorghum, maize and barley is the same as that found on the susceptible wheat cultivar McNair (Chapter 2). Niks (1986) showed that a number of rust species have a characteristic morphology of infection structures, irrespective of the plant in which they had formed, and that these characteristics could be used to identify rust taxa of single sporelings at least to the species level in the absence of the host.

As the research for Chapter 2 was conducted at the same time and under the same conditions as the components of the study presented here, comparisons of observations on and in host and non-host plants could validly be made. Heath (1974; 1977) observed that the surface characteristics of non-host leaves, such as hirsuteness and waxiness, may play an important practical part in non-host resistance in the field as these may reduce the number of urediospores which encounter favourable conditions for germination.

On the three non-hosts species in the present study, germ tubes grew in a direction perpendicular to the long axis of the leaf, then forming appressorium identical in morphology to those observed on McNair (Chapter 2). Directional growth towards a stoma, and the subsequent induction of an appressorium seems to be a response to the particular topographical features of the leaf surface (Wynn & Staples, 1981). According to Heath (1977), whether the behaviour of a rust propagule on the leaf surface plays a significant role in non-host resistance, essentially depends on whether such behaviour results in fewer attempts at penetration into the leaf than are found on the host plant. Leath & Rowell (1966) found no differences in attempts at leaf penetration by *Puccinia graminis* on wheat or maize, and an analysis of resistance components by Niks & Dekens (1987) indicated that stomatal penetration on an inappropriate (non-) host species by *Puccinia recondita* f.sp. *tritici* and *Puccinia recondita recondita* is not hampered significantly. Ferreira & Rijkenberg (1989) found that many of the germ tubes of gladiolus rust

aborted on maize leaves, and of those that successfully formed appressoria on maize, many were unable to penetrate the stomatal slit. Heath (1974, 1977) observed a reduction in penetration attempts in only certain non-host species and she concluded that reduced penetration may be an important source of resistance of at least some non-host plants in the field.

The results presented here indicate that resistance within leaves expresses itself at different times in each non-host species. The internal restriction mechanisms of sorghum showed fungal development to have been arrested at the primary infection hypha stage without the cutting off of a HMC. Later sampling revealed that the fungus does not develop beyond this. Heath (1977) found that in non-host plants, fungal growth commonly ceased before the formation of a primary HMC, and this cessation in growth did not appear to be the result of the presence of a growth inhibitor, but rather the result of the absence of a septum delimiting the HMC. In the present investigation, the absence of HMCs on hyphae in sorghum would indicate that in this non-host, *Puccinia graminis* f.sp. *tritici* does not "recognize" the environment and is not stimulated to form the septum which would delimit a HMC from the primary infection hypha.

In maize at 12 hpi however, primary infection hyphae with associated HMCs were observed, and at 24 hpi, secondary infection hyphae were present. Secondary infection hyphae constituted the most advanced stage observed on maize. Leath & Rowell (1966) recorded that HMCs were not formed in maize leaves infected with wheat stem rust, and these two authors (Leath & Rowell, 1966; Leath & Rowell, 1969; Leath & Rowell, 1970) proposed that the presence of a growth inhibitor could account for the resistance of maize to wheat stem rust. Heath (1974), investigating the growth of cowpea rust (*Uromyces phaseoli* (pers.) Wint. var *vignae* (Barcl.) Arth.) in a number of non-host plants, observed that in non-host plants in which HMCs formed, very few haustoria were seen, and ultrastructural investigations suggested that haustorium formation could be inhibited by at least three mechanisms: depostion of osmiophilic material on adjacent non-host walls, loss of contact between HMC and non-host cell, or fungal death prior to haustorium

initiation. Heath (1977) working with non-host interactions of maize, sunflower and cowpea rusts, found that whether a haustorium formed or not, secondary hyphae sometimes started to develop from the region of the infection hyphae adjacent to the HMC. These secondary infection hyphae remained short and never developed HMCs or haustoria of their own. The observations of *Puccinia graminis* f.sp. *tritici* in the non-host maize made in the present study are similar to those made by Heath (1977).

The development of *Puccinia graminis* f.sp. *tritici* on the barley cultivar Diamant, was more advanced than that on the susceptible host McNair at 12 hpi, and the secondary infection hyphae observed in barley were well developed, many with a HMC. There is no sporulation of *Puccinia graminis* f.sp. *graminis* on barley cultivar Diamant (personal observation by author), thus, clearly this barley cultivar is resistant to race 2SA2 of this pathogen. A light microscopy study of the development of *Puccinia graminis* f.sp. *tritici* on resistant and susceptible barley cultivars by Sellam & Wilcoxson (1976) revealed that there were no significant differences between resistant and susceptible cultivars in urediospore germination, appressorium formation or penetration however, growth of the pathogen was restricted in leaves of resistant, but not susceptible cultivars. From the results of the present study, it would appear that resistance to *Puccinia graminis* f.sp. *tritici* race 2SA4 is expressed in a similar manner to that shown by the resistant barley cultivars examined by Sellam & Wilcoxson (1976), ie. expressed after penetration has occurred and once the hyphae have begun to colonize the leaf tissue. It was not possible to explain why the counts of infection structures at 12 hpi in barley were higher than counts made at 24 and 48 hpi.

Heath (1982) stated that non-host responses typically occur during the earliest stages of infection and are usually characterized by the cessation of fungal growth before the formation of the first haustorium. Thus from the observations presented in this paper and in previous investigations, it can be concluded that sorghum and maize are typical non-hosts of *Puccinia graminis* f.sp. *tritici* whereas barley can be placed in the host range of this rust fungus.

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CHAPTER 4

EXPRESSION OF STEM RUST RESISTANCE GENE *Sr5*

INTRODUCTION

Histological studies have played an important rôle in the elucidation of the timing and expression of mechanisms in by plants which confer resistance to infection by the rust fungi. They have enabled researchers to determine whether the response is one of a host or a non-host plant (Niks, 1987), whether a non-host reaction is based on some kind of avoidance or on true resistance (Niks, 1981) and, whether resistance in plants to the rust fungi is expressed pre-haustorially or post-haustorially. Heath (1982b) concluded that non-host reactions to rusts are usually of the pre-haustorial type, whereas major-genic host resistance to the rusts is often post-haustorial.

A number of comparative histological light microscopy investigations have been conducted in an attempt to relate histological observations of wheat (*Triticum aestivum* L.) - stem rust (*Puccinia graminis* (Pers.) f.sp. *tritici* Erikks. & Henn.) interactions to specific stem rust (*Sr*) genes known to be present in the wheat cultivar. Rohringer *et al.* (1979) reported differences in response to infection among nearly-isogenic lines of wheat containing *Sr5, Sr6, Sr8* and *Sr22* genes for resistance to stem rust, while a number of researchers have investigated the effect of temperature sensitive *Sr* genes in wheat lines, for example, *Sr6* (Harder *et al.* 1979 a, b; Manocha, 1975; Mayama *et al.*, 1975; Samborski *et al.*, 1977; Skipp & Samborski, 1974; Skipp *et al.* 1974), *Sr15* (Gousseau & Deverall, 1986; Gousseau *et al.*, 1985), *Sr9b* and *Sr14* (Gousseau *et al.*, 1985)

The aim of this investigation was to determine the timing and expression of resistance conditioned by the stem rust resistance gene *Sr5*, and use was made of a quantitative histological technique to study the pre-penetration, penetration, and post-penetration phases of infection of race 2SA2 of the wheat stem rust fungus

formed on and in the wheat line I*Sr5*Ra compared with those formed on the wheat line I*Sr8*Ra.

MATERIALS AND METHODS

Plant material and inoculation

The wheat (*Triticum aestivum* L.) lines used were I*Sr5*Ra (C.I. 14159) and I*Sr8*Ra (C.I. 14167) which have stem rust resistance genes *Sr5* and *Sr8* respectively (Roelfs & McVey, 1979). Seedlings were grown and inoculated with race 2SA2 of the wheat stem rust fungus *Puccinia graminis* f.sp. *tritici* as described in Chapter 2. Race 2SA2 (standard race 21) has an avirulence/ virulence formula of 5, 6, 8b, 9b, 9e, 13, 17, 21, 22, 24, 25, 26, 27, 29, 30, 31, 32, 35, 36, dp2, Tt3/ 7a, 7b, 8a, 9a, 9d, 9f, 9g, 10, 11, 14, 16, 18, 19, 20, 28 (Le Roux, 1986) and gives a highly resistant infection type (0;) on I*Sr5*Ra and a fully susceptible infection type (4) on I*Sr8*Ra on the Stakman scale (Stakman *et al.*, 1962).

Ten rust-infected primary leaves of each line were collected at 48 hours post inoculation (hpi). Leaves were cut into 3 cm lengths, fixed, and stained with a 0,1% solution of the optical brightener Blancophor® BA 267% (Bayer, South Africa) as described by Kuck *et al.* (1981) and Rohringer *et al.* (1977). A previous study (Lennox & Rijkenberg, 1989) on the usefulness of a number of fluorochromes in the visualization of *Puccinia graminis* f.sp. *tritici* in wheat, revealed that Blancophor allowed for a better visualization of fungal infection structures than did Calcofluor® White ST (American Cyanamid Company, New Jersey). Consequently, Blancophor was used in the present investigation.

The leaf pieces were examined using a Zeiss research microscope fitted with epifluorescence equipment (light source HBO 50, red suppression filter BG 38, exciter filter BP 390-440, chromatic beam splitter FT 460, barrier filter LP475). Colour photographs were taken using Ektachrome 160 Professional 35 mm film.

At each infection site, the stage to which the fungal infection had advanced was

noted and, if present, the number of secondary haustorial mother cells were recorded.

Modifications to the method of Rohringer *et al.* (1977) by Kuck *et al.* (1981) for visualizing rust haustoria failed to stain haustoria reliably in the present investigation, limitations also noted by Southerton & Deverall (1989). Consequently, numbers of haustoria were not quantified in the present investigation.

Four replicates in time of the experiment were conducted and the results were compared statistically using a one-way and multivariate ANOVA test.

RESULTS

Infection types

Primary leaf infection types recorded 12 days post inoculation as described by Stakman *et al.* (1962) were 0; on I*Sr5*Ra and 4 on I*Sr8*Ra.

Fluorescence microscopy observations and analysis of data

Rust development in lines I*Sr5*Ra and I*Sr8*Ra followed the same pattern as that found in the universal stem rust susceptible wheat cultivar McNair and described in Chapter 2 of this thesis. Germ tubes, appressoria, substomatal vesicles, primary infection hyphae, secondary infection hyphae and haustorial mother cells fluoresced a bright yellow. Haustorial mother cells exhibited a much more intense fluorescence than that observed in the other fungal structures. Occasionally, haustoria were detected as small brightly fluorescing structures within the host cell. In this study, as in the fluorescence microscopy observations of stem rust in the universal stem rust susceptible wheat cultivar McNair (Lennox & Rijkenberg, 1989), the terminal cells of primary infection hyphae were seen to be haustorial mother cells, as haustoria were detected in the host cells onto which these terminal cells abutted.

Table 1 Fluorescence microscopy counts of infection structure stages of *Puccinia graminis* f.sp. *tritici* on and in two isogenic wheat cultivars at 48 hpi

CATEGORY	ISOGENIC WHEAT LINE	
	I*Sr5*Ra	I*Sr8*Ra
Germ tubes	26.55* a**	28.22 a
Appressoria not over stoma	6.46 a	7.75 a
Appressoria over stoma	48.23 a	53.35 a
Substomatal vesicle	6.63 a	7.58 a
Primary infection hypha with primary haustorial mother cell	17.50 a	18.45 a
Secondary haustorial mother cells	4.55 a	5.48 a
Total number of secondary haustorial mother cells	10.50 a	15.77 a

* Mean values calculated from counts obtained from 10 leaves in four replicates (refer to Appendix 4.1)

** Values across rows with different letters differed significantly at the P=0.01 level

Counts of pre- and post-penetration infection structures recorded in each of ten leaves of the two wheat lines, and in four replicates in time are recorded in Appendix 4.1. The data were statistically analysed and the results are presented in Table 1.

No statistically significant differences were found in pre-penetration infection structure stages between the two wheat lines, although the means of counts in I*Sr5*Ra were always lower than those in I*Sr8*Ra.

Table 2 Development of *Puccinia graminis* f.sp. *tritici* in two isogenic wheat lines at 48 hpi. The size of the colonies is characterized by the number of HMCs.

CATEGORY	ISOGENIC WHEAT LINES	
	I*Sr5*Ra	I*Sr8*Ra
No. of colonies with*** :-		
One secondary haustorial mother cell	11.50* a**	6.50 a
Two secondary haustorial mother cells	15.50 a	14.75 a
Three secondary haustorial mother cells	12.75 a	18.75 a
Four secondary haustorial mother cells	5.00 a	10.50 a
Five secondary haustorial mother cells	0.25 a	1.75 b
Six secondary haustorial mother cells	0.50 a	2.00 a
No. of colonies with*** :-		
One or two secondary haustorial mother cells	13.50 a	10.60 a
Three to six secondary haustorial mother cells	4.60 a	8.25 a

* Mean number of colonies with n secondary haustorial mother cells, where this mean value is calculated from the total number of colonies with n secondary haustorial mother cells from 10 leaves in four replicates (refer to Appendix 4.2)

** Values across rows with different letters differed significantly at the P=0.01 level

*** No significant differences when counts converted to percentage of total number of colonies per trial

Rust colonies in I*Sr5*Ra were typically associated with autofluorescing host cells which fluoresced an orange-yellow colour, whereas cells of I*Sr8*Ra were seldomly seen to exhibit this autofluorescence. Uninfected host cells showed a slight green fluorescence. Autofluorescing host cells were not quantified in this investigation.

Statistical analysis of post-penetration infection structure stages also showed that there were no significant differences in counts of these stages between the two wheat lines, however, means of counts were lower in I*Sr5*Ra.

From visual observations it appeared that I*Sr5*Ra housed fewer infection sites (or colonies) with higher numbers of secondary haustorial mother cells than I*Sr8*Ra. A comparison of the numbers of colonies and the number of secondary haustorial mother cells originating from them was made between the two wheat lines (Table 2). The two wheat lines differed significantly in only one category, namely I*Sr5*Ra had significantly lower numbers of colonies with five haustorial mother cells, although caution must be exercised in in determining the significance of this finding as the numbers of colonies with five or six haustorial mother cells were very low in both wheat lines at 48 hpi.

A study of the results in Table 2 indicate a distribution of the rust population into two distinct groups of colonies. The first group consisted of colonies with one or two secondary haustorial mother cells. There were higher numbers of this group in I*Sr5*Ra (Table 2) than in I*Sr8*Ra, although the difference was not statistically higher. The second group consisted of colonies with three to six secondary haustorial mother cells. Higher numbers (although not significantly higher) of this group were found in I*Sr8*Ra (Table 2).

A comparison of the pooled results of colonies with one to two secondary haustorial mother cells, and three to six secondary haustorial mother cells (Table 2) revealed that although I*Sr5*Ra had higher numbers of colonies with one to two secondary haustorial mother cells, and I*Sr8*Ra had higher numbers of colonies with three to six secondary haustorial mother cells, the numbers did not differ significantly.

DISCUSSION

With rust infections, it is commonly recognized that there is usually considerable variation in the behaviour of the fungus and the host at different sites in any one tissue, but that resistance genes seem to increase the frequencies of certain types of responses dramatically (Heath, 1982a; Niks, 1990). Most types of responses however, are found on most plant genotypes to some extent (Niks, 1990). Heath (1982a) cautions that the common practice of analysing data averaged from many infection sites may serve to obscure relationships between host response and fungal growth.

The appearance of hypersensitive flecks (IT 0;) in the I*Sr5*Ra - race 2SA2 interaction indicated that growth of the fungus had been restricted and host damage was minimal in this interaction. The I*Sr8*Ra - race 2SA2 interaction resulted in large sporulating pustules and could be classified as a fully susceptible interaction (IT 4). Luig & Rajaram (1972) noted that the expressions of *Sr 5* and *Sr8* are stable at temperatures normally encountered in the glasshouse, and that high temperatures did not influence the resistance expression of *Sr5* in the genetic backgrounds of Reliance and Kanred.

Previous studies have shown that the expression of resistance of *Sr5* is altered by the genetic background of the wheat host. In wheat cultivars Reliance, Prelude and Marquis, *Sr5* gave a 0 (immune) reaction type, whereas in Chinese Spring macroscopically visible flecks (IT 0;) were seen (Rohringer *et al.* (1979). Tiburzy *et al.* (1990) noted that in Prelude, *Sr5* conditioned a 0; IT, whereas in cultivars with *Sr5* and additional resistance genes (*Sr6*, *Sr7a*, *Sr9g*, *Sr22*) a 0 or immune reaction resulted.

The results presented in this paper indicated that, based on the characteristics examined, there are no significant differences between the two lines up to, and including, 48 hpi, by which time race 2SA2 had successfully formed secondary haustorial mother cells in both of these lines. These results are in keeping with

those of Tiburzy *et al.* (1990) who found that the effect of *Sr5* on the fungus is not expressed significantly until after 48 hpi. Rohringer *et al.* (1979) on the other hand, found that resistance conditioned by *Sr5* was significantly expressed by 24 hpi irrespective of the background of the host. Here it must be noted that Rohringer *et al.* (1979) used *Puccinia graminis* f.sp. *tritici* race C17, Tiburzy *et al.* (1990) race 32, and the present investigation made use of race 2SA2. The differences in timing of expression of *Sr5* are possibly due to the different host-cultivar/pathogen-race combinations used in each study.

The present investigation's results are in keeping with the results of other researchers working on major resistance gene effects (Heath, 1982b), in that these major genes for resistance do not affect the development of the fungus prior to the establishment of the primary haustorium. Heath (1982b) stated that this is a common, although not universal, finding in rust-host interactions.

A number of pre-haustorial effects on rust development have been documented however (Heath, 1982b), and the work of Tani *et al.* (1975) is of particular interest. These researchers found that pre-haustorial elongation of infection hyphae was retarded in an incompatible oat-rust combination.

With major-gene resistance, resistance is usually first expressed when the first host cell is invaded, ie. with the formation of the first haustorium (Heath, 1981). Tiburzy *et al.* (1990) found that in the resistant wheat line Prelude-*Sr5*, which gives a 0; reaction type, inhibition of fungal growth was not detected before the first haustorium was formed, but occurred after the hypersensitive reaction of the host cells that were penetrated by the first haustoria.

The species-specific form of a substomatal infection structure pre-determines the host cell or tissue type that is preferentially penetrated by the first haustorium. In many rust species the first cell penetrated is a mesophyll cell (Tiburzy *et al.* 1990). This does not seem to be the case in wheat-stem-rust interactions, as Skipp & Samborski (1974) investigating the *Sr6*/P6 interaction, found that 34 to 49% of all

penetrants formed the first haustorium in an epidermal cell, and Tiburzy *et al.* (1990) observed that in wheat lines with *Sr5*, epidermal cells were penetrated by the first haustorium in up to 95% of all infection sites, whereas fewer than 3% of the infection sites had haustoria in mesophyll cells. Scanning electron microscopy observations of *Puccinia graminis* f.sp. *tritici* in the universal susceptible wheat cultivar McNair, presented in Chapter 2 of this thesis, indicated that the first haustorial mother cell commonly forms abutting onto an epidermal cell adjacent to one of the swollen ends of the guard cells

The autofluorescence of host cells in the I*Sr5*Ra-race 2SA2 interaction is indicative of the hypersensitive response (HR) conditioned by a number of major resistance genes (Rohringer *et al.*, 1979). Fluorescing host cells in a resistant *Sr5* interaction were assumed to be necrotic (Rohringer *et al.*, 1979), an assumption supported by the ultrastructural investigation of the *Sr5*- wheat stem rust interaction described by Harder *et al.* (1979a) and Harder *et al.* (1979b). In the present investigation, the virtual absence of autofluorescence of infected host cells in the susceptible I*Sr8*Ra-race 2SA2 interaction is similar to the observation by Tiburzy *et al.* (1990) that infected susceptible host cells did not fluoresce in the time period 20 to 40 hpi. Hence, with *Sr5*, it would appear that autofluorescence is an indication of incompatibility.

In the literature there has been much debate over the significance of the hypersensitive response (host necrosis) in the expression of major-gene resistance to rust fungi (Heath, 1976; Király & Barna, 1985; Király *et al.*, 1972). The results of some investigations have been interpreted as indicating that necrosis has a primary rôle in resistance (Heath 1982a; Jones & Deverall, 1977; Keen & Littlefield, 1979; Maclean *et al.*, 1974; Samborski *et al.*, 1977; Skipp & Samborski, 1974), while others have been suggested to show that necrosis is not mandatory for resistance or that it may be a consequence rather than the cause of the cessation of fungal growth (Barna *et al.*, 1974; Brown *et al.*, 1966; Campbell & Deverall, 1980; Király & Barna, 1985; Király *et al.*, 1972; Mayama *et al.*, 1975; Ogle & Brown, 1971). Rohringer *et al.* (1979) found that colony inhibition in resistant

wheat possessing *Sr5* was not significantly associated with fluorescing host cells, and as such host cell necrosis is not correlated with inhibition of fungal growth in this interaction, whereas Tiburzy *et al.* (1990) showed that inhibition of the fungus was closely associated with autofluorescence of the infected epidermal cell and concluded that the hypersensitive reaction is closely associated with resistance controlled by the *Sr5* gene, and is possibly the determining factor. Heath (1982a) stated that the fact that there is no simple relationship between fungal growth and the amount of necrosis in some types of rust resistance may not necessarily imply that necrosis has no importance in restricting fungal development and concluded that rather than host necrosis *per se*, it may be the timing of necrosis relative to haustorium development, and the effect of this necrosis on haustorium function, which is critical in determining the rôle of such necrosis in host resistance to rust fungi. Bushnell (1982), after reviewing the available evidence, concluded that each resistance gene conditions incompatibility in a different unique way, that is, the amount of tissue involved and the amount of fungus growth varies depending upon the gene conditioning incompatibility.

Beardmore *et al.* (1983) used a number of techniques in an effort to characterize the autofluorescing compounds found in resistant wheat cultivars, in particular those with *Sr5* and *Sr6*. Their results indicated an initial phenolic accumulation followed by lignification of the whole cell contents and the authors stated that such cells are irreversibly changed with loss of viability, collapse and contraction, and the reaction forms an incompatible ring of necrotic cells around the penetration site. Tiburzy & Reisener (1990) levelled the following criticism at the work of Beardmore *et al.* (1983): they had examined the accumulation of compounds in necrotic mesophyll cells in an advanced stage of the infection process, whereas Tiburzy *et al.* (1990) had found that resistance based on the *Sr5* gene is first expressed in epidermal cells as early as 32 hpi, and that mesophyll cells were completely unpenetrated at many infection sites. Using autoradiographic and histochemical tests, Tiburzy & Reisener (1990) determined that there was an accumulation of polymeric phenolics, lignin or lignin-like material and callose in autofluorescing necrotic cells of wheat with resistance based on the *Sr5* gene. They also found a correlation between the

inhibition of synthesis of lignin or lignin-like polymers and reduced resistance, and suggested that this supports the hypothesis that cellular lignification is an important factor in resistance in this system.

Bushnell (1982) stated that in the stem rust - wheat interaction, *Sr5* is the only studied gene that commonly gives a determinant hypersensitivity reaction i.e. the hypersensitive response leads to the complete halt in fungus growth. This author referred to the work of Rohringer *et al.* (1979) as an illustration of the determinant hypersensitivity reaction expressed by *Sr5*, and noted that this response is only found in certain genetic backgrounds. The results of Tiburzy *et al.* (1990) indicated that *Sr5* in the wheat line Prelude had a determinant hypersensitivity reaction when inoculated with stem rust race 32, as growth of the fungus was completely inhibited three days post-inoculation.

Sr5 in Prelude or in Chinese Spring backgrounds had a significant effect on the linear growth of the rust colonies of race C17 as early as 24 hpi (Rohringer *et al.*, 1979) whereas Tiburzy *et al.* (1990) found that *Sr5* had a significant effect on the colony growth of race 32 in the wheat cultivar Prelude three days after inoculation, an effect which resulted in the cessation of fungal growth in the resistant line. A higher resolution time course study of fungal development by Tiburzy *et al.* (1990) revealed that inhibition of fungal growth was apparent at 32 hpi. Once again, the specific host-cultivar/pathogen-race combinations are possibly the cause of variation in expression of resistance.

Tiburzy *et al.* (1990) and Rohringer *et al.* (1979) used the number of haustorial mother cells to characterise the size of a colony, and Tiburzy *et al.* (1990) found that over a six day period the rust population in a resistant cultivar with *Sr5* was distributed into two distinct groups of colonies namely, those with one to three haustorial mother cells and those with more than five haustorial mother cells. The majority of the colonies with one to three haustorial mother cells were associated with intensely fluorescing epidermal cells, and haustoria in these cells remained small and spherical. Fluoresecence of infected mesophyll cells occurred in about

one third of these infection sites and most of the colonies with more than five haustorial mother cells were associated with fluorescing mesophyll cells and no, or only faint, fluorescence in infected epidermal cells.

In the present study, I*Sr5*Ra appeared to house infection sites with smaller numbers of secondary haustorial mother cells than did I*Sr8*Ra and statistical analysis of counts revealed that I*Sr8*Ra had significantly higher numbers of colonies with five secondary haustorial mother cells. The importance of this difference is somewhat debatable as both lines had very low numbers of colonies with five and six secondary haustorial mother cells. A grouping of the colonies with secondary haustorial mother cells into those with one or two , and those with three to six secondary haustorial mother cells, revealed that I*Sr5*Ra had higher numbers of colonies with one or two secondary haustorial mother cells, whereas I*Sr8*Ra had higher numbers of colonies with three to six secondary haustorial mother cells, although these numbers were not significantly higher. Thus it appeared that *Sr5* had some influence on the fungus at 48 hpi.

Rohringer *et al.* (1979) found that the host genetic background determined the number of haustorial mother cells found in rust colonies in cultivars with *Sr5*-dependent rust resistance. In Prelude, Marquis and Reliance backgrounds, few colonies developed more than two haustorial mother cells, whereas in Chinese Spring background, one third of the colonies had developed more than five haustorial mother cells at 72 hpi.

Inhibition of haustorial development was correlated with the intensity of fluorescence of necrotic epidermal cells (Tiburzy *et al.* 1990). Intensely fluorescing epidermal cells contained small spherical haustoria, the growth of which was not terminated before the haustorial bodies had reached a size of about 4μm in diameter, whereas weakly fluorescing cells contained haustoria that were intermediate in size between those in intensely fluorescing cells and those in susceptible host cells.

Growth of the colony and the number of secondary haustorial mother cells in

incompatible interactions was closely correlated with the state of the first haustorium, in that colonies with a medium-sized or large first haustorium developed more than five secondary haustorial mother cells, whereas hypha associated with a small first haustorium in an intensely fluorescing epidermal cell were inhibited after the differentiation of one, two or three secondary haustorial mother cells (Tiburzy *et al.*, 1990).

It is generally unclear how the necrosis detected by light microscope techniques relates to the various stages of cellular disorganization visualized under the electron microscope (Heath, 1982b). Rohringer *et al.* (1979) and Tiburzy *et al.* (1990) reported that in the *Sr5*/P5 interaction, both mesophyll and epidermal cells fluoresced when invaded by an avirulent race, although Rohringer *et al.* (1979) noted that the fluorescing epidermal cells were not collapsed. Transmission electron microscopy investigations of the incompatible interaction of race C17 in wheat cultivar Marquis (*Sr5*) in epidermal (Harder *et al.*, 1979a) and mesophyll (Harder *et al.*, 1979b) cells revealed that infected epidermal and mesophyll cells were necrotic. Necrosis of infected epidermal cells was detected at 36 hpi (ie. 24 hours after the end of the 12 hour dark period) and usually occurred during the early expansion phase of the development of the first haustorium. Haustoria in necrotic epidermal cells usually remained limited in size (3 - 4 μm in diameter). In their fluorescence microscopy investigation of race 32 in Prelude-*Sr5*, Tiburzy *et al.* (1990) also noted that the growth of haustoria in fluorescing epidermal cells was not terminated before haustorial bodies had reached a size of about 4 μm in diameter. This, they said, reflects the minimum time required to develop the resistance response from its induction to its deleterious effect on the haustorium. Harder *et al.* (1979a) noted that symptoms of haustorial disorganization in epidermal cells involved premature vacuolation of the haustoria and irregularities in the sheath structure and that where haustorial necrosis occurred, epidermal cell necrosis was also present.

Resistance expression of *Sr6* has been studied extensively by a number of researchers using both light and electron microscopy techniques (Kim *et al.*, 1977;

Harder *et al.*, 1979 a,b; Manocha, 1975; Mayama *et al.*, 1975; Samborski *et al.*, 1977; Skipp & Samborski, 1974; Skipp *et al.*, 1974) and investigations have shown that incompatibility in leaves containing this gene is expressed in mesophyll cells only (Rohringer *et al.*, 1979; Harder *et al.*, 1979a). Intracellular symptoms of incompatibility in mesophyll cells of *Sr5*/P5 interactions were found by Harder *et al.* (1979b) to be similar to those of the *Sr6*/P6 interactions. In host cells possessing either *Sr5* or *Sr6*, early ultrastructural symptoms of incompatibility were a more electron-dense and often perforated invaginated host-plasmalemma, disruptions of the non-invaginated host-plasmalemma, vacuolation of the cytoplasm, and accumulations of electron-dense material along the membranes of the vacuoles. A gradual increase in the size of electron-dense accumulations along vacuole membranes, and chloroplast and mitochondrial membranes followed, and ultimately, the entire protoplast was electron-dense and collapsed. Necrosis of fungal tissue followed a different pattern from that of host cells in that incompatibility in haustoria was usually first expressed by a uniform increase in electron density of the protoplast, which eventually obscured the organelles. Incompatibility was usually expressed in haustoria before it became evident in the associated haustorial mother cells.

Harder *et al.* (1979b) noted that haustorial necrosis commonly occurred in association with, or was evident before there was any indication of host necrosis, although in a few instances, an invaded host cell was necrotic without evidence of disorganization in the associated haustorium. They stated that products from a necrotic haustorium or a necrotic cell do not appear to be responsible for necrosis of the other participant of the interaction.

The interaction I*Sr5*Ra - race 2SA2 fulfils Flor's gene-for- gene hypothesis, a situation which implies a differential interaction between the race of the pathogen and the host cultivar (Van der Plank, 1975). This interaction also implies the recognition of a specific fungal product (an elicitor) by a host receptor (Callow, 1984), with recognition being controlled by the gene for resistance in the plant (Keen, 1982). From the observations of Tiburzy *et al.* (1990), Harder *et al.*

(1979a) and Harder *et al.* (1979b) that the first signs of incompatibility in *Sr5*-dependent resistant cultivars commonly occurs once a haustorium has been formed in the host cell, it would appear that the recognition between the two reacting partners takes place at the plasma membrane. A number of researchers working on a variety of gene-for-gene interactions have come to a similar conclusion as to the site of recognition in these interactions (Callow, 1984; Keen, 1982). Differentiation of a haustorial mother cell and initiation of host cell penetration is the start of the "parasitic phase" of a rust infection, the phase in which most race-specific interactions leading to compatibility or incompatibility are expressed, and the phase at which host defences begin in incompatible interactions (Mendgen *et al.*, 1988).

The zone of interaction between intracellular host-parasite surfaces has been found to be complex and highly specialized (Littlefield & Heath, 1979) and although the structure of this zone is becoming better known (Chong & Harder, 1982; Chong *et al.*, 1986; Knauf *et al.*, 1989; Plotnikova *et al.*, 1979), less is known of the chemical components involved. This paucity of information is due to the fact that conventional methods of biochemical analysis have not proved useful in the elucidation of the biochemistry of interactions at the host-parasite interface in rust diseases. What is required are more precise methods which locate chemical components and/or changes at the intracellular level (Harder & Mendgen, 1982). Kogel *et al.* (1984) identified galactolipid receptors on the outer surface of wheat plasma membranes which specifically bound certain lectins, and Kogel *et al.* (1985) found evidence for the direct involvement of these galactoconjugates in the process of host-parasite recognition.

Reisener *et al.* (1986) reported that from extracts of germinated uredospores of *Puccinia graminis* f.sp. *tritici* they isolated a fraction that was able to elicit the characteristic hypersensitive response in an *Sr5*-dependent resistant wheat line. The elicitor showed a differential effect when tested against *Sr5* and *sr5* near-isogenic lines. They stated that the elicitor is most probably a glycoprotein.

Glycoproteins related to specificity were identified in intercellular washing fluids from stem rust-affected wheat leaves (Rohringer & Martens, 1987) and Harder *et al.* (1989) stated that one possible *in vivo* location of these glycoproteins is the fungal cell surface, however conventional processing for electron microscopy has not proved useful in demonstrating the materials that may coat the surface of rust fungus cells which ramify intercellularly in the host.

The use of novel techniques has shed some light on the nature of extramural materials of intercellular fungal cells. Mendgen *et al.* (1985), using the binding properties of lectins and enzymes were able to determine that substomatal vesicles and infection hyphae of *Puccinia coronata* and *Uromyces appendiculatus* have mainly glucans on their outer surfaces. Making use of a variety of tissue-processing techniques, Harder *et al.* (1989) were able to demonstrate the presence of considerable amounts of extramural material occurring in several different configurations on rust fungus intercellular hyphae. They concluded that rôles of the components in specificity related to compatibility or incompatibility, and to adhesion, remain to be elucidated.

Cessation of fungal growth may not be primarily due to shortage of nutrients but may result from effects of antifungal compounds such as phytoalexins. However, with wheat there is as yet little evidence for the existence of such compounds (Tiburzy *et al.*, 1990) and Reisener *et al.*, (1986) stated that it is highly unlikely that phytoalexins are involved in the expression of the *Sr5* resistance response.

Brodny *et al.* (1986) investigating the residual and interactive expression of "defeated" wheat stem rust resistance genes, concluded that *Sr6*, *Sr8* and *Sr9a* each has a residual expression when confronted by matching virulence genes. This residual expression of *Sr8* in I*Sr8*Ra would reduce the differences in counts between I*Sr5*Ra and I*Sr8*Ra and hence affect the interpretation of resistance expressed by *Sr5* in I*Sr5*Ra.

From the results of the present investigation, and those of previous studies

presented in this discussion, the following sequence of events in the expression of the *Sr5* gene at an infection site can be concluded: (i) formation of pre-penetration infection structures without inhibition; (ii) formation of a substomatal vesicle, primary infection hypha, and primary haustorial mother cell without inhibition; (iii) penetration of a host cell {epidermal or mesophyll} and the formation of the first haustorium; (iv) recognition by the resistance gene of the host cell of an elicitor from the avirulent pathogen; (v) inhibition of the expansion growth of the haustorium and initiation of necrosis of the haustorium; (vi) necrosis of the infected host cell accompanied by an accumulation of lignin or lignin-like compounds; (vii) inhibition or cessation of growth of intercellular hyphae and a restriction in the number of secondary haustorial mother cells.

The timing and expression of resistance conditioned by *Sr5* is influenced by the host cultivar and the race of the pathogen and it is essential that this is taken into account when comparing the results of investigations into the expression of resistance conditioned by this gene.

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Soybean callus yield (g/flask) obtained for the kinetin standards (μg/l) run simulaneously with each bioassay

	Kinetin (μg/l)	Callus yield* (g/flask)	P = 0.01
Experiment 1			
Bioassay of Paper chromatography fractions	0	0.147	0.174
	1	0.243	
	10	0.331	
	50	0.483	
Bioassay of HPLC fractions	0	0.316	0.378
	1	0.279	
	10	0.291	
	50	0.344	
Experiment 2			
Bioassay of Column chromatography fractions	0	0.355	0.487
	1	0.380	
	10	0.819	
	50	1.245	
Bioassay of HPLC fractions	0	0.058	0.099
	1	0.055	
	10	0.078	
	50	0.211	

* Mean mass of 3 flasks

Positions of authentic cytokinin markers as measured by UV absorbance at 265nm on HPLC

CYTOKININ	RETENTION TIME (minutes)
Ade	6
Ado	18
Z9G	24
ZOG	26
tZ	32
DHZ	33
tZR	52
DHZR	59
2iP9G	66
2iP	74
iPA	84

Soybean callus bioassay of primary leaf and seed material of Little Club and Little Club Sr25 using Paper Chromatography and High Pressure Liquid Chromatography

Table 1 Callus yield (g/flask) of fractions obtained from paper chromatography separation of primary leaf (2.5 g) and seed (1 g) material of Little Club and Little Club *Sr25*

Rf	Little Club		Little Club *Sr25*	
	Leaf	Seed	Leaf	Seed
0.1	0.120	0.151	0.022	0.286
0.2	0.112	0.193	0.130	0.243
0.3	0.051	0.270	0.152	0.175
0.4	0.145	0.213	0.074	0.197
0.5	0.098	0.123	0.142	0.155
0.6	0.228	0.313	0.334	0.512
0.7	0.400	0.196	0.156	0.194
0.8	0.356	0.178	0.136	0.217
0.9	0.392	0.160	0.072	0.277
1.0	0.278	0.172	0.247	0.256

Table 2 Callus yield (g/flask) of fractions obtained from HPLC separation of primary leaf material (0.3125 g) of Little Club and Little Club *Sr25* previously separated by paper chromatography

Elution time	Little Club			Little Club *Sr25*		
	Rf	Rf		Rf	Rf*	
	0.1-0.5	0.6-1.0	Pool	0.1-0.5	0.6-1.0	Pool
1	0.729	0.089	0.818	0.357		
2	0.427	0.215	0.642	0.638		
3	0.365	0.077	0.442	0.671		
4	0.227	0.039	0.266	1.082		
5	0.284	0.179	0.463	0.475		
6	0.741	0.260	1.001	0.477		
7	0.545	0.045	0.590	0.569		
8	0.275	0.072	0.347	0.668		
9	0.293	0.165	0.458	0.458		
10	0.224	0.049	0.273	0.260		
11	0.180	0.198	0.378	0.686		
12	0.415	0.284	0.699	0.800		
13	0.720	0.079	0.799	0.384		
14	0.450	0.107	0.557	0.451		
15	0.397	0.244	0.641	0.918		
16	0.489	0.089	0.678	0.679		
17	0.078	0.311	0.389	0.893		
18	0.226	0.264	0.490	1.055		
19	0.459	0.041	0.500	1.025		
20	0.376	0.044	0.420	0.736		
21	0.255	0.058	0.313	0.821		
22	0.476	0.089	0.565	0.800		
23	0.265	0.118	0.383	0.551		
24	0.302	0.074	0.376	0.726		
25	0.334	0.177	0.511	0.686		
26	0.258	0.077	0.335	0.768		
27	0.440	0.105	0.545	0.675		
28	0.504	0.099	0.435	0.611		
29	0.324	0.147	0.471	0.704		
30	0.181	0.356	0.537	0.488		
31	0.298	0.237	0.535	0.424		
32	0.237	0.354	0.591	0.796		
33	0.680	0.130	0.810	0.849		
34	0.305	0.239	0.344	0.749		
35	0.337	0.992	1.329	0.724		
36	0.509	0.220	0.729	0.250		
37	0.383	0.967	1.350	0.821		
38	0.250	0.873	1.125	0.282		
39	0.223	0.470	0.693	0.631		
40	0.341	0.906	1.247	0.278		
41	0.205	1.162	1.367	0.271		
42	0.640	0.205	0.845	0.339		
43	0.251	0.646	0.897	0.744		
44	0.179	0.641	0.820	0.422		
45	0.326	0.747	1.073	0.289		

Table 2 Continued

Callus yield (g/flask) of fractions obtained from HPLC separation of primary leaf material (0.3125 g) of Little Club and Little Club Sr25 previously separated by paper chromatography

Elution time	Little Club			Little Club Sr25		
	Rf 0.1-0.5	Rf 0.6-1.0	Pool	Rf 0.1-0.5	Rf 0.6-1.0	Pool
46	0.325	0.030	0.355	0.455		
47	1.044	0.697	1.741	0.805		
48	0.145	0.869	1.014	0.468		
49	0.231	0.565	0.796	0.673		
50	0.454	0.327	0.781	0.595		
51	0.499	0.634	1.133	0.658		
52	0.354	0.672	1.026	1.601		
53	0.241	0.819	1.060	0.921		
54	0.244	0.024	0.268	0.421		
55	0.111	0.383	0.494	0.713		
56	0.165	0.544	0.709	0.426		
57	0.353	1.198	15516	1.018		
58	0.163	0.526	0.689	0.647		
59	0.222	0.519	0.741	0.633		
60	0.287	0.823	1.110	0.656		
61	0.200	0.689	0.889	0.689		
62	0.461	0.338	0.799	0.426		
63	0.486	0.520	1.006	0.409		
64	0.331	0.383	0.714	0.610		
65	0.730	0.438	1.168	0.521		
66	0.487	0.534	1.021	0.566		
67	0.374	0.547	0.921	0.459		
68	0.836	0.431	1.267	0.448		
69	0.622	0.350	0.972	0.782		
70	0.520	0.718	1.238	0.701		
71	0.383	0.905	1.288	0.697		
72	0.688	0.800	1.488	0.847		
73	0.584	0.693	1.277	0.741		
74	0.209	0.855	1.064	0.573		
75	0.900	1.372	2.272	0.666		
76	1.048	1.200	2.248	1.271		
77	0.297	0.646	0.943	0.578		
78	0.525	1.362	1.887	1.009		
79	0.372	0.826	1.198	0.594		
80	0.201	1.049	1.250	0.295		
81	0.344	0.307	0.651	0.396		
82	0.261	1.292	1.553	0.976		
83	0.450	0.879	1.329	0.560		
84	0.222	0.935	1.157	0.584		
85	0.600	0.388	0.988	0.054		
86	0.577	0.859	1.436	0.092		
87	0.232	0.764	0.996	0.042		
88	0.264	0.404	0.668	1.059		
89	0.725	0.652	1.377	0.554		
90	0.444	0.367	0.811	0.527		

* This extract was lost during preparation for HPLC

Table 3 Callus yield (g/flask) of fractions obtained from HPLC separation of seed material (0.125 g) of Little Club and Little Club *Sr25* previously separated by paper chromatography

Elution time	Little Club			Little Club *Sr25*		
	Rf 0.1-0.5	Rf 0.6-1.0	Pool	Rf 0.1-0.5	Rf 0.6-1.0	Pool
1	0.105	0.056	0.161	0.288	0.939	1.227
2	0.172	0.048	0.220	0.443	0.247	0.690
3	0.093	0.048	0.141	0.244	0.451	0.695
4	0.054	0.039	0.093	0.633	0.447	1.080
5	0.957	0.056	1.013	0.213	0.561	0.774
6	0.047	0.048	0.095	0.333	0.627	0.960
7	0.913	0.061	0.974	0.058	1.193	1.251
8	0.228	0.082	0.310	0.096	0.498	0.594
9	0.136	0.074	0.210	0.068	0.722	0.790
10	0.883	0.044	0.927	0.353	0.196	0.549
11	0.286	0.067	0.353	0.790	1.154	1.944
12	0.135	0.043	0.178	0.400	0.726	1.126
13	0.0	0.066	0.066	0.470	0.448	0.918
14	0.075	0.053	0.128	0.293	0.327	0.620
15	0.053	0.052	0.105	0.221	0.690	0.911
16	0.079	0.159	0.238	0.271	0.353	0.624
17	0.070	0.042	0.112	0.303	0.337	0.640
18	0.047	0.356	0.403	0.175	0.277	0.452
19	0.101	0.124	0.225	0.425	0.636	1.061
20	0.122	0.419	0.541	0.098	0.506	0.604
21	0.057	0.151	0.208	0.416	0.690	1.106
22	0.061	0.182	0.243	0.783	0.928	1.711
23	0.062	0.157	0.219	0.549	0.639	1.188
24	0.030	0.064	0.094	0.303	0.631	0.934
25	0.468	0.066	0.534	0.388	0.403	0.791
26	0.532	0.089	0.621	0.253	0.356	0.609
27	0.726	0.058	0.784	0.431	0.645	1.076
28	0.678	0.057	0.735	0.316	1.025	1.341
29	0.660	0.103	0.763	0.228	0.915	1.143
30	0.055	0.465	0.520	1.068	0.733	1.801
31	0.086	0.773	0.859	0.472	0.372	0.844
32	0.019	0.265	0.284	0.400	1.047	1.447
33	0.067	0.250	0.317	0.412	1.057	1.469
34	0.071	0.605	0.676	0.320	1.071	1.391
35	0.063	0.778	0.841	0.164	0.673	0.837
36	0.075	0.227	0.302	0.325	0.857	1.182
37	0.060	0.174	0.234	0.168	1.086	1.254
38	0.072	0.229	0.301	0.556	0.610	1.166
39	0.022	0.716	0.738	0.196	1.004	1.200
40	1.173	0.479	1.652	0.276	0.578	0.854
41	0.043	0.717	0.760	0.448	0.168	0.616
42	0.073	0.614	0.687	0.436	0.131	0.567
43	0.077	0.727	0.804	0.886	0.091	0.977
44	0.903	1.352	2.255	0.198	0.385	0.583
45	0.106	0.946	1.052	0.401	0.614	1.015

Table 3 Continued Callus yield (g/flask) of fractions obtained from HPLC separation of seed material (0.125 g) of Little Club and Little Club *Sr25* previously separated by paper chromatography

Elution time	Little Club			Little Club *Sr25*		
	Rf 0.1-0.5	Rf 0.6-1.0	Pool	Rf 0.1-0.5	Rf 0.6-1.0	Pool
46	0.207	0.686	0.893	0.182	0.427	0.609
47	1.399	1.273	2.672	0.277	1.027	1.304
48	0.079	1.196	1.275	0.141	0.216	0.357
49	0.038	1.032	1.070	0.417	1.240	1.657
50	0.080	0.428	0.508	0.070	0.617	0.687
51	0.044	0.203	0.247	0.084	0.575	0.659
52	0.055	0.336	0.391	0.068	0.258	0.326
53	0.052	0.590	0.642	0.046	0.733	0.779
54	0.061	0.210	0.271	0.054	0.185	0.239
55	0.057	0.551	0.608	0.057	1.215	1.272
56	0.058	0.419	0.477	0.311	0.754	1.065
57	0.069	1.279	1.348	0.080	0.743	0.823
58	0.045	0.987	1.032	0.113	0.611	0.652
59	0.044	1.108	1.152	0.039	0.857	0.896
60	0.077	0.578	0.655	0.059	0.471	0.530
61	0.126	0.731	0.857	0.043	0.138	0.181
62	0.150	0.461	0.611	0.995	0.846	1.841
63	0.130	0.570	0.700	0.170	0.693	0.863
64	0.098	0.376	0.474	0.106	0.958	1.064
65	0.081	0.481	0.562	0.048	0.655	0.703
66	0.704	0.948	1.652	0.067	1.034	1.101
67	0.074	1.022	1.096	0.195	0.271	0.466
68	0.092	1.066	1.158	0.490	0.150	0.640
69	0.087	1.007	1.094	0.211	0.728	0.939
70	0.203	0.642	0.845	0.047	0.982	1.029
71	0.534	0.628	1.162	0.113	1.378	1.491
72	0.199	0.441	0.640	0.094	0.306	0.400
73	0.049	0.330	0.379	0.129	0.138	0.267
74	0.057	0.689	0.746	0.066	0.405	0.471
75	0.076	0.120	0.196	0.055	0.305	0.360
76	0.062	0.472	0.534	0.041	0.277	0.318
77	0.035	0.899	0.934	0.293	0.283	0.522
78	0.048	0.331	0.379	0.061	0.243	0.304
79	0.031	0.632	0.663	0.067	0.165	0.232
80	0.075	0.0	0.075	0.482	0.173	0.655
81	0.094	0.618	0.712	0.489	0.764	1.253
82	0.098	0.599	0.697	0.039	0.058	0.097
83	0.052	0.417	0.469	0.084	0.165	0.249
84	0.365	0.381	0.746	0.243	0.156	0.399
85	0.066	0.335	0.401	0.060	0.267	0.327
86	0.151	0.152	0.303	0.045	0.158	0.193
87	0.048	0.508	0.556	0.094	0.209	0.303
88	0.047	0.060	0.107	0.417	0.052	0.469
89	0.107	0.351	0.458	0.749	0.053	0.802
90	0.073	0.319	0.392	0.048	0.228	0.276

Soybean callus bioassay of primary leaf and seed material of Little Club and Little Club Sr25 separated using Sephadex Column Chromatography followed by HPLC separation of the column chromatography fractions

Table 1 Callus yield (g/flask) of fractions obtained by Sephadex separation of leaf material (1.25 g)

Elution volume	Little Club			Little Club Sr25		
	Rep A	Rep B	Mean	Rep A	Rep B	Mean
40	0.461	0.432	0.447	0.284	0.338	0.311
80	0.493	0.709	0.601	0.753	0.662	0.708
120	0.869	0.504	0.687	1.013	1.173	1.093
160	0.985	0.133	0.559	0.342	0.812	0.577
200	0.325	0.290	0.308	0.601	0.108	0.355
240	0.047	0.032	0.040	0.196	0.214	0.205
280	0.242	0.077	0.160	0.164	0.161	0.163
320	0.164	0.224	0.194	0.298	0.177	0.238
360	0.144	0.404	0.274	0.385	0.301	0.343
400	0.227	0.233	0.230	0.880	0.351	0.616
440	0.234	0.410	0.322	1.171	0.584	0.878
480	0.393	0.781	0.587	0.489	1.005	0.747
520	0.0	0.148	0.074	0.895	0.258	0.577
560	0.301	0.207	0.254	0.348	0.295	0.322
600	0.487	0.174	0.331	0.607	0.208	0.408
640	0.674	0.316	0.495	0.763	0.141	0.452
680	0.737	0.171	0.454	1.026	0.779	0.903
720	0.457	0.183	0.320	0.516	0.688	0.602
760	0.444	0.311	0.378	0.391	0.378	0.385
800	0.587	0.282	0.435	0.512	0.087	0.300
840	0.466	0.197	0.332	0.824	0.677	0.751
880	0.255	0.521	0.388	1.001	0.424	0.713
920	0.319	0.249	0.284	0.609	0.834	0.722
960	0.433	0.204	0.319	0.735	0.315	0.525
1000	0.567	0.228	0.398	0.489	0.660	0.575
1040	0.843	0.408	0.626	0.679	0.303	0.491
1080	0.390	0.357	0.374	0.547	0.314	0.431
1120	0.131	0.330	0.231	0.304	0.457	0.381
1160	0.104	0.308	0.206	1.069	0.422	0.746
1200	1.115	0.163	0.639	0.537	0.320	0.429
1240	0.483	0.519	0.501	0.854	0.197	0.526
1280	0.822	0.303	0.563	0.630	0.0	0.315
1320	0.425	0.271	0.348	0.465	0.425	0.445
1360	0.152	0.661	0.407	0.800	0.455	0.628
1400	0.540	0.220	0.380	0.134	0.261	0.198
1440	2.153	0.968	1.561	0.740	0.624	0.682
1480	0.374	0.071	0.223	0.272	0.743	0.508
1520	0.244	0.235	0.240	0.367	0.459	0.413
1560	0.263	0.214	0.239	0.887	0.097	0.492
1600	0.339	0.491	0.415	0.847	0.399	0.623

Table 2 Callus yield (g/flask) of fractions obtained by Sephadex separation of seed material (0.5 g)

Elution volume	Little Club			Little Club *Sr25*		
	Rep A	Rep B	Mean	Rep A	Rep B	Mean
40	0.570	0.407	0.489	0.482	0.163	0.323
80	0.259	0.342	0.301	0.396	0.613	0.505
120	0.863	0.713	0.788	0.570	0.236	0.403
160	0.319	0.811	0.565	0.511	0.220	0.366
200	0.117	0.205	0.161	0.190	0.076	0.133
240	0.311	0.734	0.523	0.431	0.560	0.496
280	0.387	0.151	0.269	0.188	0.090	0.139
320	0.455	0.261	0.403	0.258	0.206	0.232
360	0.352	1.272	0.812	0.475	0.376	0.426
400	0.430	0.326	0.378	0.572	0.119	0.346
440	0.525	0.483	0.504	0.465	0.483	0.474
480	0.248	0.521	0.385	0.346	0.379	0.363
520	0.272	0.519	0.396	0.591	0.687	0.639
560	0.314	0.886	0.600	0.290	0.444	0.367
600	0.676	0.308	0.492	0.156	0.549	0.353
640	0.689	0.656	0.673	0.176	0.122	0.149
680	0.164	0.486	0.325	0.208	0.102	0.155
720	0.826	0.509	0.668	0.430	0.213	0.322
760	0.234	0.749	0.492	0.322	0.543	0.433
800	0.610	0.604	0.607	0.215	0.611	0.413
840	0.202	0.418	0.310	0.275	0.207	0.241
880	0.386	0.226	0.306	0.074	0.237	0.156
920	0.320	0.254	0.287	0.459	0.256	0.358
960	0.440	0.509	0.475	0.496	0.409	0.453
1000	0.282	0.390	0.336	0.511	0.344	0.428
1040	0.704	0.367	0.536	0.646	0.420	0.533
1080	0.234	0.447	0.341	0.615	0.661	0.638
1120	0.483	0.336	0.410	0.420	0.253	0.337
1160	0.163	0.128	0.146	0.282	0.264	0.273
1200	0.359	0.322	0.341	0.338	0.415	0.377
1240	1.041	0.738	0.890	0.403	0.342	0.373
1280	0.457	0.656	0.557	0.435	0.347	0.391
1320	0.616	0.518	0.567	0.255	1.043	0.649
1360	0.568	0.201	0.385	0.443	0.263	0.353
1400	0.388	0.302	0.345	0.274	0.527	0.401
1440	0.362	0.247	0.305	0.367	0.458	0.413
1480	0.293	0.484	0.389	0.244	0.358	0.301
1520	0.528	0.572	0.550	0.541	0.444	0.493
1560	0.335	0.270	0.303	0.577	0.362	0.470
1600	0.403	0.407	0.405	0.101	0.182	0.142

Table 3 Callus yield (g/flask) of fractions obtained by HPLC separation of column chromatography fractions of leaf material of Little Club

Elution time	Elution volume (ml)					Pooled data
	0-200	200-520	520-760	760-1000	1000-1600	
2	0.026	0.021	0.063	0.091	0.0	0.201
4	0.048	0.017	0.065	0.080	0.041	0.251
6	0.043	0.229	0.099	0.062	0.098	0.531
8	0.031	0.072	0.057	0.197	0.019	0.376
10	0.066	0.064	0.051	0.088	0.065	0.334
12	0.049	0.019	0.101	0.022	0.038	0.229
14	0.050	0.054	0.089	0.214	0.199	0.606
16	0.046	0.042	0.052	0.072	0.028	0.240
18	0.050	0.043	0.077	0.107	0.127	0.404
20	0.025	0.036	0.040	0.066	0.058	0.225
22	0.034	0.027	0.094	0.079	0.018	0.255
24	0.040	0.012	0.093	0.088	0.032	0.265
26	0.054	0.114	0.056	0.134	0.058	0.416
28	0.052	0.036	0.111	0.021	0.052	0.272
30	0.017	0.032	0.111	0.117	0.047	0.324
32	0.034	0.039	0.077	0.098	0.039	0.287
34	0.024	0.021	0.057	0.184	0.150	0.436
36	0.030	0.040	0.106	0.079	0.192	0.447
38	0.042	0.025	0.077	0.128	0.022	0.294
40	0.083	0.029	0.058	0.046	0.062	0.278
42	0.077	0.013	0.087	0.119	0.028	0.324
44	0.060	0.089	0.120	0.072	0.084	0.425
46	0.043	0.100	0.050	0.073	0.062	0.328
48	0.042	0.050	0.052	0.081	0.047	0.272
50	0.053	0.028	0.081	0.109	0.109	0.380
52	0.056	0.028	0.080	0.139	0.024	0.327
54	0.028	0.007	0.051	0.041	0.067	0.194
56	0.050	0.037	0.101	0.061	0.052	0.113
58	0.042	0.029	0.070	0.099	0.019	0.259
60	0.044	0.028	0.064	0.118	0.041	0.295
62	0.041	0.010	0.045	0.115	0.050	0.261
64	0.080	0.010	0.129	0.155	0.043	0.417
66	0.032	0.052	0.042	0.892	0.043	1.064
68	0.071	0.026	0.054	0.108	0.017	0.276
70	0.083	0.014	0.086	0.098	0.114	0.395
72	0.050	0.020	0.072	0.117	0.048	0.307
74	0.025	0.013	0.071	0.102	0.260	0.271
76	0.044	0.047	0.077	0.122	0.146	0.436
78	0.049	0.027	0.070	0.223	0.011	0.380
80	0.054	0.042	0.066	0.061	0.032	0.255
82	0.050	0.027	0.071	0.042	0.073	0.263
84	0.049	0.026	0.075	0.099	0.607	0.856
86	0.110	0.046	0.035	0.107	0.034	0.332
88	0.098	0.036	0.284	0.089	0.027	0.534
90	0.085	0.038	0.079	0.196	0.068	0.467

Table 4 Callus yield (g/flask) of fractions obtained by HPLC separation of column chromatography fractions of leaf material of Little Club *Sr25*

Elution time	Elution volume (ml)					Pooled data
	0-200	200-520	520-760	760-1000	1000-1600	
2	0.053	0.064	0.088	0.039	0.012	0.256
4	0.077	0.027	0.028	0.075	0.057	0.507
6	0.084	0.016	0.138	0.056	0.074	0.368
8	0.037	0.046	0.071	0.108	0.020	0.282
10	0.048	0.0	0.094	0.049	0.035	0.226
12	0.017	0.031	0.072	0.075	0.058	0.253
14	0.057	0.056	0.070	0.036	0.059	0.258
16	0.0	0.062	0.082	0.075	0.048	0.267
18	0.090	0.118	0.067	0.095	0.078	0.448
20	0.048	0.037	0.064	0.069	0.023	0.241
22	0.0	0.030	0.125	0.052	0.039	0.246
24	0.014	0.012	0.122	0.056	0.041	1.118
26	0.051	0.128	0.074	0.157	0.138	0.548
28	0.040	0.043	0.064	0.078	0.034	0.259
30	0.068	0.014	0.114	0.099	0.101	0.396
32	0.094	0.047	0.028	0.349	0.053	0.571
34	0.035	0.054	0.144	0.345	0.055	0.633
36	0.075	0.043	0.061	0.219	0.076	0.474
38	0.048	0.028	0.121	0.090	0.0	0.287
40	0.062	0.061	0.054	0.104	0.036	0.308
42	0.086	0.044	0.019	0.135	0.058	0.342
44	0.006	0.041	0.069	0.061	0.028	0.205
46	0.017	0.055	0.081	0.063	0.037	0.253
48	0.083	0.032	0.062	0.046	0.080	0.303
50	0.048	0.065	0.117	0.069	0.043	0.342
52	0.056	0.056	0.062	0.094	0.107	0.375
54	0.042	0.053	0.070	0.067	0.055	0.287
56	0.013	0.050	0.047	0.065	0.038	0.213
58	0.019	0.020	0.060	0.092	0.079	0.270
60	0.053	0.066	0.092	0.408	0.0	0.619
62	0.049	0.064	0.072	0.069	0.101	0.355
64	0.047	0.051	0.100	0.047	0.059	0.304
66	0.040	0.069	0.039	0.077	0.049	0.274
68	0.062	0.029	0.089	0.054	0.039	0.273
70	0.042	0.113	0.020	0.056	0.024	0.255
72	0.044	0.017	0.062	0.051	0.112	0.286
74	0.032	0.061	1.017	0.063	0.032	1.205
76	0.0	0.059	0.096	0.081	0.134	0.370
78	0.033	0.062	0.101	0.103	0.089	0.388
80	0.042	0.050	0.055	0.090	0.076	0.313
82	0.056	0.052	0.046	0.081	0.031	0.266
84	0.025	0.085	0.037	0.090	0.265	0.502
86	0.011	0.026	0.072	0.075	0.083	0.267
88	0.035	0.030	0.061	0.121	0.045	0.292
90	0.014	0.059	0.061	0.085	0.058	0.277

Table 5 Callus yield (g/flask) of fractions obtained by HPLC separation of column chromatography fractions of seed material of Little Club

Elution time	Elution volume (ml)					Pooled data
	0-200	200-520	520-760	760-1000	1000-1600	
2	0.049	0.034	0.032	0.032	0.033	0.180
4	0.059	0.014	0.060	0.034	0.026	0.193
6	0.170	0.014	0.030	0.019	0.030	0.263
8	0.015	0.024	0.023	0.013	0.049	0.124
10	0.059	0.037	0.021	0.019	0.057	0.193
12	0.098	0.023	0.029	0.013	0.049	0.212
14	0.046	0.054	0.041	0.020	0.042	0.203
16	0.037	0.093	0.020	0.029	0.036	0.215
18	0.088	0.010	0.038	0.017	0.074	0.227
20	0.041	0.039	0.007	0.014	0.037	0.138
22	0.073	0.022	0.027	0.027	0.058	0.207
24	0.053	0.017	0.027	0.047	0.071	0.215
26	0.075	0.050	0.031	0.027	0.071	0.233
28	0.058	0.058	0.053	0.012	0.061	0.242
30	0.052	0.027	0.021	0.044	0.028	0.172
32	0.094	0.014	0.045	0.023	0.045	0.221
34	0.054	0.083	0.026	0.038	0.041	0.242
36	0.019	0.017	0.033	0.021	0.044	0.134
38	0.056	0.061	0.030	0.026	0.037	0.210
40	0.054	0.060	0.023	0.020	0.038	0.195
42	0.027	0.040	0.063	0.011	0.037	0.178
44	0.072	0.033	0.023	0.031	0.074	0.233
46	0.104	0.020	0.030	0.020	0.043	0.217
48	0.054	0.020	0.032	0.034	0.092	0.232
50	0.070	0.060	0.009	0.008	0.075	0.222
52	0.126	0.042	0.019	0.032	0.083	0.302
54	0.072	0.027	0.029	0.025	0.042	0.195
56	0.029	0.025	0.034	0.017	0.041	0.146
58	0.080	0.029	0.034	0.036	0.036	0.215
60	0.022	0.034	0.024	0.007	0.044	0.131
62	0.074	0.043	0.010	0.040	0.054	0.221
64	0.030	0.008	0.009	0.027	0.085	0.159
66	0.055	0.028	0.011	0.032	0.049	0.175
68	0.296	0.017	0.018	0.037	0.079	0.447
70	0.054	0.031	0.036	0.042	0.062	0.225
72	0.052	0.028	0.034	0.018	0.055	0.187
74	0.111	0.040	0.034	0.027	0.070	0.282
76	0.058	0.048	0.013	0.038	0.084	0.241
78	0.073	0.012	0.039	0.027	0.044	0.191
80	0.018	0.028	0.023	0.017	0.084	0.170
82	0.119	0.041	0.025	0.027	0.036	0.248
84	0.041	0.041	0.021	0.009	0.0	0.112
86	0.057	0.024	0.008	0.034	0.048	0.171
88	0.093	0.041	0.037	0.021	0.058	0.250
90	0.077	0.020	0.030	0.011	0.046	0.184

Table 6 Callus yield (g/flask) of fractions obtained by HPLC separation of column chromatography fractions of seed material of Little Club *Sr25*

Elution time	Elution volume (ml)					Pooled data
	0-200	200-520	520-760	760-1000	1000-1600	
2	0.024	0.024	0.043	0.008	0.042	0.141
4	0.048	0.032	0.018	0.049	0.095	0.242
6	0.039	0.083	0.012	0.043	0.091	0.268
8	0.008	0.031	0.029	0.019	0.116	0.203
10	0.079	0.033	0.027	0.022	0.105	0.266
12	0.084	0.033	0.013	0.045	0.078	0.253
14	0.028	0.039	0.020	0.025	0.108	0.220
16	0.104	0.039	0.037	0.031	0.078	0.289
18	0.104	0.032	0.029	0.027	0.045	0.237
20	0.030	0.012	0.070	0.043	0.075	0.167
22	0.068	0.032	0.019	0.032	0.038	0.189
24	0.045	0.027	0.036	0.025	0.059	0.192
26	0.067	0.038	0.047	0.036	0.061	0.249
28	0.066	0.034	0.011	0.030	0.052	0.193
30	0.080	0.011	0.030	0.056	0.040	0.217
32	0.152	0.046	0.015	0.032	0.050	0.295
34	0.045	0.023	0.050	0.032	0.060	0.210
36	0.059	0.046	0.052	0.067	0.082	0.306
38	0.079	0.031	0.018	0.044	0.050	0.222
40	0.105	0.083	0.040	0.031	0.047	0.306
42	0.027	0.038	0.006	0.036	0.057	0.164
44	0.074	0.020	0.023	0.021	0.031	0.169
46	0.100	0.038	0.017	0.010	0.053	0.218
48	0.074	0.029	0.020	0.025	0.074	0.222
50	0.086	0.039	0.022	0.015	0.075	0.237
52	0.065	0.032	0.023	0.020	0.054	0.194
54	0.354	0.060	0.059	0.022	0.078	0.573
56	0.062	0.027	0.021	0.007	0.057	0.174
58	0.052	0.012	0.031	0.021	0.024	0.140
60	0.067	0.027	0.012	0.023	0.102	0.231
62	0.028	0.046	0.034	0.041	0.065	0.214
64	0.039	0.061	0.023	0.009	0.039	0.171
66	0.072	0.023	0.024	0.030	0.055	0.204
68	0.060	0.042	0.040	0.029	0.072	0.243
70	0.186	0.060	0.017	0.031	0.069	0.363
72	0.070	0.043	0.030	0.018	0.051	0.212
74	0.056	0.041	0.039	0.025	0.048	0.209
76	0.115	0.024	0.041	0.064	0.060	0.304
78	0.105	0.009	0.015	0.023	0.051	0.203
80	0.056	0.036	0.033	0.034	0.055	0.214
82	0.066	0.025	0.039	0.023	0.075	0.228
84	0.169	0.035	0.025	0.044	0.058	0.331
86	0.110	0.036	0.012	0.062	0.066	0.286
88	0.094	0.047	0.017	0.036	0.0	0.194
90	0.057	0.036	0.014	0.034	0.067	0.208

APPENDIX 2.1

Counts of infection structures of *Puccinia graminis tritici* that had developed to the indicated levels on wheat cv. McNair at specific time intervals post inoculation

INFECTION STRUCTURE LEVEL	HOURS-POST-INOCULATION (hpi)			
	12	24	48	96
Ovoid substomatal vesicle	60	12	62	54
Collapsed ovoid substomatal vesicle	7	1	3	14
Spherical substamatal vesicle	9	11	11	19
Collapsed spherical substomatal vesicle	1	2	17	48
Atypical primary infection hypha	-	-	6	1
Primary infection hypha	8	12	4	3
Primary infection hypha with haustorial mother cell	17	35	13	14
Secondary infection hypha	-	47	21	27
Intercellular mycelium with haustorial mother cells	-	3	44	123
n = number of sites observed	102	123	181	303

Counts of infection structures of *Puccinia graminis tritici* that had developed to the indicated levels on maize, sorghum and barley at specific time intervals post-inoculation

INFECTION STRUCTURE LEVEL	HOURS-POST-INOCULATION (hpi)											
	SORGHUM				MAIZE				BARLEY			
	12	24	48	96	12	24	48	96	12	24	48	96
Ovoid substomatal vesicle	14	42	33	13	82	7	11	3	40	46	48	11
Collapsed ovoid substomatal vesicle	-	-	-	3	-	-	2	-	5	2	29	1
Spherical substomatal vesicle	13	18	-	3	12	3	6	2	15	14	7	1
Collapsed spherical substomatal vesicle	-	7	-	-	1	-	4	-	34	1	26	3
Atypical primary infection hypha	-	6	-	2	-	14	13	1	2	4	21	1
Primary infection hypha	10	2	14	7	43	41	47	12	9	28	20	4
Primary infection hypha with haustorial mother cell					15	7	7	-	20	54	57	3
Secondary infection hypha					-	1	4	4	39	55	9	2
Intercellular mycelium with haustorial mother cell									72	8	3	29
n = number of sites observed	37	75	47	30	153	73	94	22	236	212	220	55

Counts of infection structures of *Puccinia graminis* f,sp. *tritici* race 2SA2 that had developed to the indicated levels on I*Sr5*Ra and I*Sr8*Ra by 48 hpi.

I*Sr5*Ra

CATEGORY	REP	LEAF NUMBER										X*	X**
		1	2	3	4	5	6	7	8	9	10		
Germ tubes	1	5	18	37	11	13	21	21	30	23	32	21.1	
	2	34	57	34	47	30	32	48	54	30	47	38.3	
	3	19	16	29	19	14	28	16	19	17	31	20.8	
	4	14	35	9	34	45	36	35	4	13	35	26.0	26.55
Appressoria not over stoma	1	2	1	11	3	2	3	11	2	6	14	4.5	
	2	6	22	8	17	18	10	10	23	7	7	12.8	
	3	5	6	6	9	7	4	3	3	1	3	4.7	
	4	3	3	8	2	3	5	6	1	3	5	3.9	6.46
Appressoria over stoma	1	13	21	38	26	35	22	53	27	22	31	28.8	
	2	40	80	46	64	114	29	71	70	47	75	63.6	
	3	54	29	84	36	30	76	29	48	67	41	49.4	
	4	44	68	26	52	53	38	107	38	14	71	51.1	48.23
Substomatal vesicle	1	6	1	8	3	11	5	19	0	6	34	9.3	
	2	30	4	11	3	3	1	10	2	11	6	8.1	
	3	1	1	12	2	8	2	9	2	2	2	4.1	
	4	12	6	0	8	4	4	12	4	0	0	5.0	6.63
Primary infection hypha with primary haustorial mother cell	1	33	5	34	4	8	20	22	10	1	27	16.4	
	2	44	11	34	61	7	39	88	3	28	60	37.5	
	3	3	14	2	2	9	3	10	15	0	6	6.4	
	4	29	12	5	6	19	19	6	2	8	1	9.7	17.50
Secondary haustorial mother cells	1	0	0	0	0	0	3	0	0	0	0	0.3	
	2	6	0	7	7	2	7	26	1	13	7	7.6	
	3	1	17	5	0	4	0	4	16	1	3	5.1	
	4	6	8	3	2	10	8	1	2	11	1	5.2	4.55
Total number of secondary haustorial mother cells	1						7					0.7	
	2	9	0	16	17	4	15	49	1	22	16	14.9	
	3	1	40	13	0	12	0	8	52	1	9	13.6	
	4	15	16	9	6	24	15	2	7	33	1	12.8	10.50

X* Mean of counts from 10 leaves

X** Overall mean of four replicates

I*Sr8*Ra

CATEGORY	REP	LEAF NUMBER										X*	X**
		1	2	3	4	5	6	7	8	9	10		
Germ tubes	1	38	23	20	35	6	16	14	18	33	8	19.1	
	2	21	35	29	41	24	24	71	70	61	102	47.8	
	3	39	15	15	15	12	31	23	13	36	21	20.0	
	4	17	40	21	13	45	36	31	19	24	14	26.0	28.22
Appressoria not over stoma	1	13	14	0	7	1	2	4	10	3	10	6.4	
	2	9	18	12	11	4	11	12	18	13	38	14.6	
	3	12	11	5	3	4	6	2	3	10	3	5.9	
	4	3	9	3	1	10	4	2	5	2	2	4.1	7.75
Appressoria over stoma	1	29	40	16	47	8	12	11	36	33	41	27.3	
	2	16	41	88	20	16	77	140	66	64	146	67.4	
	3	90	46	29	33	63	47	28	40	29	61	46.6	
	4	93	98	38	33	99	110	50	84	63	53	72.1	53.35
Substomatal vesicle	1	17	8	1	11	1	4	4	28	30	14	11.8	
	2	6	19	6	10	5	8	16	9	11	15	10.5	
	3	4	2	6	0	8	1	19	2	7	1	5.0	
	4	3	0	5	3	1	10	3	2	0	3	3.0	7.58
Primary infection hypha with primary haustorial mother cell	1	29	61	2	43	11	12	12	34	66	57	31.7	
	2	37	63	14	25	32	16	22	31	18	4	26.2	
	3	14	2	9	11	11	6	2	10	7	11	8.3	
	4	17	11	17	8	5	2	13	5	0	8	7.6	18.45
Secondary infection hypha	1	6	1	0	1	1	0	0	1	1	0	1.1	
	2	13	13	6	2	22	3	1	6	4	0	7.0	
	3	4	4	4	5	19	7	1	12	17	0	7.3	
	4	6	2	12	11	4	0	17	1	0	12	6.5	5.48
Total number of secondary haustorial mother cells	1	23	3	0	2	3	0	0	1	1	0	3.3	
	2	36	39	9	6	61	9	1	14	14	0	18.9	
	3	10	14	9	13	51	15	1	39	47	0	19.9	
	4	11	9	37	38	12	0	62	2	0	39	21.0	15.77

X* Mean of counts from 10 leaves

X** Overall mean of four replicates

Numbers of secondary haustorial mother cells (HMC) associated with infection sites (colonies) in four replicates of 10 leaves of I*Sr5*Ra and I*Sr8*Ra

I*Sr5*Ra

REP	Total Number of Colonies with n Haustorial Mother Cells, where n =					
	1	2	3	4	5	6
1	0	2	1	0	0	0
2	27	29	16	4	0	0
3	9	12	19	10	0	1
4	10	19	15	6	1	1
Mean	11.5	15.5	12.8	5	0.25	0.5

I*Sr8*Ra

REP	Total Number of Colonies with n Haustorial Mother Cells, where n =					
	1	2	3	4	5	6
1	2	2	4	1	1	0
2	9	18	30	11	1	1
3	10	25	20	13	3	2
4	5	14	21	17	2	5
Mean	6.5	14.8	18.8	10.5	1.8	2