PUCCINIA GRAMINIS f.sp. TRITICI, RACE C17: PHYSIOLOGY OF UREDOSPORE GERMINATION AND GERMTUBE DIFFERENTIATION

By

Sarah J. Hopkinson

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Department of Plant Science

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Germinating uredospores of race C17 of Puccinia graminis f.sp. tritici form characteristic infection structures (appressorium, infection peg, vesicle, infection hypha) in response to a 1.5 h heat shock at 29^0 C administered 2 h after germination at 19^0 C. The proportion of sporelings forming infection structures was augmented by nutrients, n-nonyl alcohol and, an appropriately timed heat shock. The heat shock temperature required to induce maximum differentiation had a very precise optimum which varied slightly for each spore lot. Variations one degree above or below this optimum reduced the percent differentiation by greater than 40%. The presence of an inhibitor of protein synthesis, puromycin, in the germination medium: (1) prevented uredosporeling differentiation but had no effect on germination, (2) significantly reduced the proportion of germtubes forming appressoria, and (3) in most cases prevented the division of germtube nuclei. It was concluded that essential differentiation-specific proteins are synthesized from the onset of germination, throughout the formation of appressoria and to the completion of differentiation. These results were consistent with the observed effects of heat shock on the rate of protein hydrolysis. During germination there was a net hydrolysis of protein leading to an increase in size of the endogenous pool of free amino acids and to an increased

leakage of amino acids to the germination medium. Heat shock effectively reduced the amount of endogenous free amino acids and the extent to which amino acids were lost to the medium. It was concluded that in heat shocked sporelings protein synthesis was increased relative to protein hydrolysis by comparison with the relative rates of these two processes in germinating (non-shocked) uredosporelings. Moreover, there was no net protein synthesis during the formation of infection structures induced by heat shock. The loss of amino acids to the germination medium was selective, particularly in heat shocked sporelings.

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1. INTRODUCTION

Rust fungi are biotrophic pathogens on plants, causing great losses to many cultivated crops. The black stem rust of wheat pathogen, Puccinia graminis Pers. f.sp. tritici Eriks and E. Henn. ranks among the more serious of cereal diseases. The stem rust is macrocyclic and heteroecious; that is, the life cycle exhibits five distinct spore stages on two hosts. The uredial stage, which is the most destructive economically, perpetuates the fungus throughout the growing season by reinfecting the cereal host.

Rust fungi that appear morphologically identical but infect different host genera are classed as formae speciales (f.sp.). Within each forma specialis there are many physiological races which are pathogenic on only certain varieties within the host species. P. graminis f.sp. tritici, race C17 (formerly race 56), was employed in the current study. The race is genetically stable with only one rare subrace 56A known. It is highly aggressive on wheat and capable of causing huge economic losses in North America.

The specific reaction of a host cultivar to a series of physiological races is determined by complementary genes for resistance in the host and virulence genes in the pathogen (i.e. by gene-for-gene reactions) (Flor 1971). In most cases incompatibility (resistant reaction) results from the interaction of a dominant gene for resistance (R) in the host

and a complimentary dominant gene for avirulence (P) in the pathogen. All other reactions (R-pp, rrP-, and rrpp) result in host-pathogen compatibility (susceptible reaction).

Infection of a host plant by the rust fungi is characterized by a series of morphological events (Figure 1). The uredospore germinates readily on water. The germtube of wheat stem rust grows across the cuticular ridges of the leaf blade; upon contact with a stomatal pore germtube elongation ceases and differentiation begins. Differentiation involves the sequential development of infection structures: the appressorium (app), the infection peg (ip), the substomatal vesicle (ssv), and the infection hypha (ih). Once established, the mycelium grows primarily in an intracellular fashion, obtaining nutrients from host cells through specialized structures called haustoria. Nutrients are also absorbed by intercellular mycelium.

The susceptible host is able to both induce or assist fungal morphogenesis and provide the balance of nutrients essential for further growth. The ability to culture the wheat rust pathogen axenically was first demonstrated using an Australian race of wheat stem rust (Williams et al. 1966). Research utilizing axenic culture can provide information regarding the biology (metabolism, nutrition, and genetics) of the rust fungi. Unfortunately, serious obstacles such as the very slow growth rates of these fungi, and their unpredictable nutritional requirements have limited the widespread use of

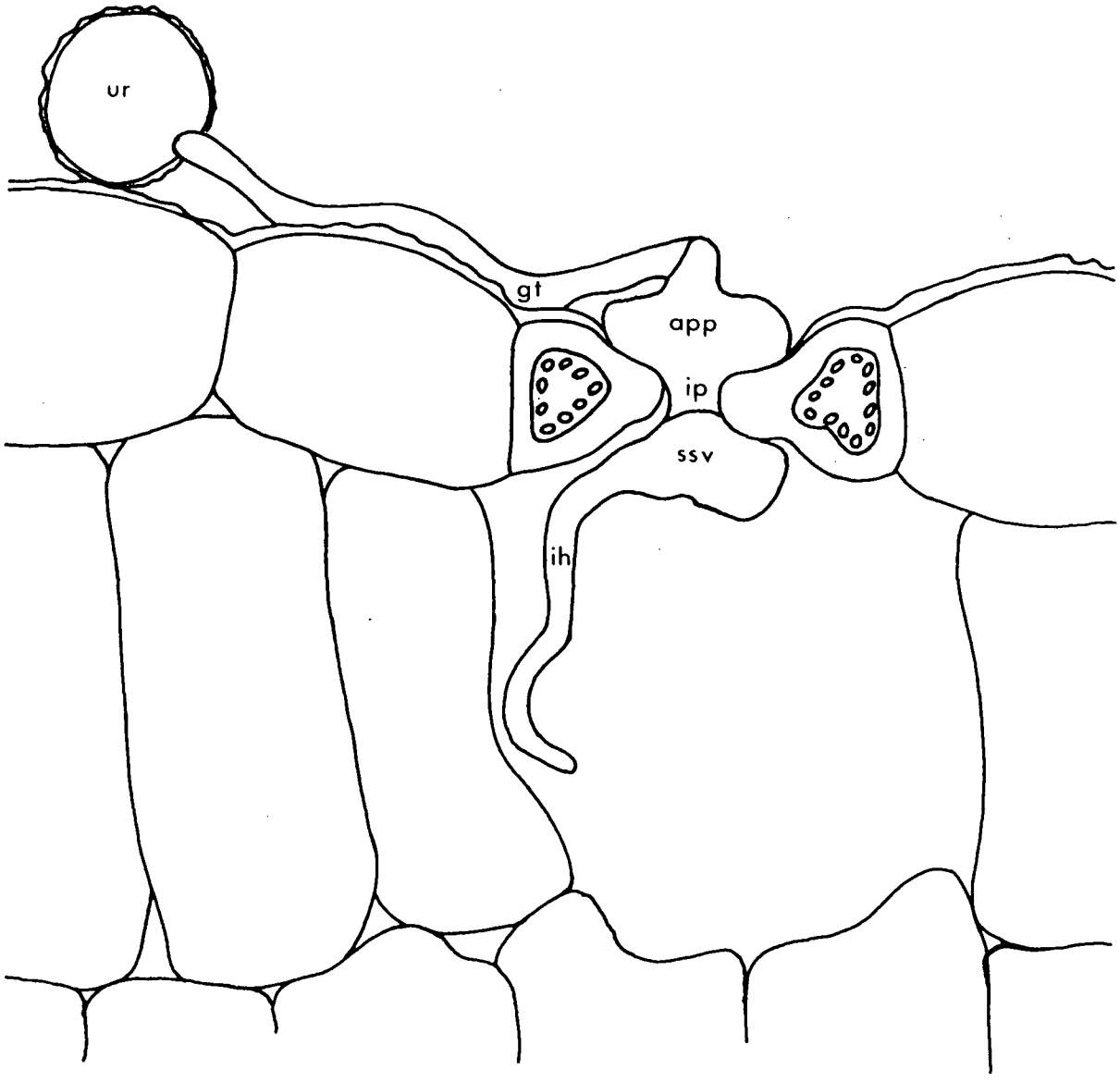


Fig. 1. Germination and differentiation of the rust pathogen, P. graminis tritici, on the cereal host: The wheat stem rust uredospore (Ur) germinates readily on the leaf surface. Contact with a stomatal pore causes the germtube (gt) to cease linear growth and begin differentiation: an appressorium (app) forms over the stomate, an infection peg (ip) grows between the guard cells and expands to form the substomatal vesicle (ssv), the vesicle gives rise to the infection hypha (ih).

axenic culture methods (Maclean 1982). Furthermore, it seems apparent, at least with P. graminis tritici, that genetically aberrant forms are encouraged to develop, that sporulation is at best erratic and that fewer than half the races that have been tested have survived under the culture conditions employed (Williams 1984).

According to Williams (1971) differentiation is a necessary prelude towards obtaining genetically normal (haploid, dikaryotic) pathogenic colonies. The proportion of germlings that form infection structures is influenced by nutrients, heat shock, the time of inoculum collection, and by the genetic constitution of the spore. Sporeling differentiation is most often induced by a timed heat shock (30^0C, 1.5 h) administered 2 h following germination at 19^0C.

In order to establish a given rust fungus in culture, specific nutritional requirements must be met. The medium must contain inorganic salts, a source of carbohydrate, reduced nitrogen, and reduced sulphur (e.g. cysteine). Howes reported that a wide range of amino compounds were excreted into culture filtrates of P. graminis tritici after 9 days incubation (see Howes and Scott 1973). The full extent of amino acid leakage during the first 20 h of germination and differentiation was not assessed. This study was designed to investigate the leakage of free amino acids from uredospore germlings concomitant with the processes of germination and differentiation (induced by heat shock). The changes in the

endogenous free amino acid pool were also assessed.

The objectives of the present study thus were:

1. To reexamine the temperature requirement for the process of differentiation of the wheat stem rust fungus.

2. To examine the influence of nutrients on the proportion of germlings that form infection structures.

3. To determine the timing of essential protein synthesis during differentiation.

4. To investigate nuclear behaviour accompanying germination and differentiation of P. graminis tritici, race C17: nuclear division, nuclear migration patterns, and nuclear distribution.

5. To determine the amount and kinds of free amino acids present during the germination of wheat stem rust uredospores (race C17) and the differentiation of germ tubes into infection structures.

    5.1 To characterize the free amino acids within nongerminated uredospores and those adhered to the outer spore wall.

    5.2 To assess the changes in the endogenous free amino acid pools of uredospore germlings during germination and differentiation.

    5.3 To determine the amounts and kinds of amino acids leached into the medium during the process of germination and differentiation.

2. LITERATURE REVIEW

2.1 Uredospore Germination and Morphogenesis

2.1.1 Germination Inhibitors and Stimulants

Uredospore germination and germtube differentiation is subject to regulating mechanisms involving endogenous inhibitors and stimulators. Germination inhibitors are dormancy agents which resemble hormones in their mobility from spore to spore, their regulatory action and their high biological activity (Allen 1976). Sporostatic levels of cis-cinnamates are found in uredospores. The effect of the inhibitors is readily reversible, for example, during hydration, and prior to germination, the active cis-cinnamates are released and are either diluted to below active levels or converted to inactive trans-isomers by ultra violet light (420 nm) (Allen 1972). The germination inhibitor of wheat stem rust is methyl cis-ferulate: a methyl ester of 3-methoxy-4-hydroxycinnamate (Macko et al. 1971). It has an ED_{50} of 0.2 ng/ml (Macko et al. 1972) and is active only during germination (mycelial growth is not affected [Allen 1976]). The inhibitor prevents the dissolution of the germ pore plug but its molecular site of action is unknown (Hess 1975).

Endogenous germination stimulants such as n-nonyl alcohol (nonanal) (French and Weintraub 1957), 6-methyl-5-hepten-2-one (Rines et al. 1974), coumarins, and phenols (Van Sumere et al.

1957) are not species specific. Macko and co-workers (1976) suggest that the stimulants facilitate germination by promoting the release of the inhibitors from the spore.

2.1.2 Thigmodifferentiation

Uredosporeling differentiation may be induced *in vitro* by physical stimuli. In a series of experiments employing bean rust germlings and artificial membranes Dickinson (1949, 1971) demonstrated that the formation of infection structures relies on a recognition event that is correlated with specific thigmotropic stimuli. Differentiation thus stimulated (thigmodifferentiation) requires firm attatchment of the germtube to the inductive surface (Wynn and Staples 1981). Recent data suggest that, in bean rust, proteins on the hyphal surface are involved in substrate adhesion and the transmission of a signal to the cytoskeleton to begin nuclear division (Epstein *et al*. 1985).

According to Hoch *et al.* (1987) the topographical signal required to trigger maximum cell differentiation in *Uromyces appendiculatus* is a 0.5 m high ridge on the substrate surface. The elevation of the ridge is critical, ridges greater than 1.0 m or less than 0.25 m are not effective signals and are unable to promote maximum differentiation. It was also reported that the growth of the germtubes is oriented by ridge spacings of 0.5 to 6.7 m.

Although collodion membranes containing paraffin oil

induced bean rust germlings to differentiate, they had little to no effect on P. g. tritici (Maheshwari et al. 1967b). Staples and his group (1983) found that contact stimuli are responsible for the induction and positioning of the wheat stem rust appressorium. However, further development of the stem rust germling requires other environmental factors most likely of host origin.

2.1.3 Thermodifferentiation

Another type of induction was reported for P. g. tritici. The exposure of germinated uredospores to elevated temperatures of 30 to 31°C for 90 min followed by the return to lower temperatures promoted up to 90% differentiation (Maheshwari et al. 1967, Dunkle and Allen 1971). The percentage of germlings that formed infection structures was influenced by the duration of heat shock, the spore to liquid ratio, and the composition of the medium present during the induction period (Dunkle and Allen 1971).

The induction of differentiation by heat shock, as described above was repeated by Wisdom (1977). The results proved unsatisfactory, yielding differentiation percentages of less than 5%. The effect of the temperature of the heat shock was not investigated. The Ca-K buffer used by Dunkle and Allen (1968) was abandoned in favour of a medium composed of Maheshwari's-buffer (Maheshwari et al. 1967), peptone, and glucose (MPG). MPG supported up to 80 percent differentiation

(Wisdom 1977).

2.1.4 Chemodifferentiation

Chemodifferentiation is the induction of infection structures by a chemical agent. Chemical stimuli include uredospore distillates such as acrolein (2-propanal) (Macko et al. 1978), the potassium ion (Staples et al. 1982), certain reduced nucleotides (Staples et al. 1983a), and volatile leaf constituents and phenolic compounds leached or metabolized from the guard cells (Grambow and Riedel 1977, Grambow 1978, Grambow and Grambow 1978). Synthetic short-chain aliphatic compounds with conjugated double bonds (e.g. acrolein) are morphogenetically active whereas saturated aliphatic aldehydes, ketones and alcohols, as well as phenylacrolein are unable to induce differentiation (Wolf 1982). The ability of a chemical agent to stimulate differentiation opens the possibility that the agent is a normal intermediary in contact-triggered differentiation (Allen 1976).

2.1.5 Mechanisms of Differentiation

The mechanisms involved in the regulation of germtube differentiation are unknown. Allen (1976) suggests that the formation of infection structures is controlled by the uredospore genome. An appropriate stimulus activates the program. The observed inhibition of differentiation by protein and RNA inhibitors suggests that the genetic message

is activated by de-repressing a region of the genome. Alternatively it is possible that the cytoskeleton (microfibrillar system) is responsible for the induction of nuclear division in bean rust germlings (Hoch and Staples 1985, Hoch et al. 1984, Staples and Hoch 1982). These authors suggest that the cytoskeleton somehow represses nuclear division until it is subjected to heat shock or an inductive membrane topography.

2.1.6 The Role of Infection Structures

In nature the infection structures play a number of important roles: Infection structures must form before leaf-colonizing hyphae can grow (Dickinson 1949, Chakravati 1966). According to Wisdom (1977), differentiated germlings of the wheat stem rust fungus infect exposed host mesophyll; undifferentiated sporelings are unable to cause infection. Infection structures serve to anchor the fungus at the stomatal site thereby enabling penetration. They represent the site of the transition from germtube to hyphal growth. This hypothesis is supported by the activation of protein, RNA and DNA synthesis during differentiation (Dunkle et al. 1969, Meihle 1972). And lastly they enable the fungus to complete the transition with the rapidity demanded by a changing (perhaps to a less conducive) environment. Grambow (1977) found that dikaryotic hyphae branching directly from germtubes are produced at a high rate but not before the third day after

seeding. Dikaryotic hyphae arise from a complete set of infection structures very soon after germination.

According to Williams (1971) the formation of infection structures is an essential prelude to the establishment and maintenance of genetically normal pathogenic colonies. The transition (parasitic to saprophytic growth), if appropriately stimulated, occurs with or without the induction of differentiated structures. However, infection structures appear to control nuclear behaviour and as a consequence increase the probability of giving rise to hyphae which are both normal and stable for the dikaryophase.

Although heat shock generally appears to stimulate cell differentiation and improve saprophytic growth of most rust fungi, Bushnell (1976) claims that heat shock does not promote growth of P. graminis f.sp. tritici, race 17. Kuck and Reisner (1985) report that differentiation has a negative effect on the in vitro sporulation of P. graminis tritici, race 32.

2.2 Cytological Events

2.2.1 Nuclear Changes

The morphological changes leading to the formation of infection structures are accompanied by a series of precisely timed nuclear events. The resting uredospore is dikaryotic haploid. Following germination, the two nuclei migrate with

the cytoplasm into the developing germtube (Heath and Heath 1978). Given the stimulus to differentiate the nuclei may divide prior to (Maheshwari et al. 1967b), or following (Grambow and Muller 1978) the development of the appressorium. Nuclear division occurs only after the linear growth of the germtube is arrested and is rarely observed in nondifferentiated germlings (Maheshwari et al. 1967, Grambow and Muller 1978, Wisdom 1977).

Dickinson (1949) reported that two or three rounds of mitotic division occur during the formation of infection structures in P. graminis and P. triticina. The appressorium may contain four to eight nuclei which migrate into the developing vesicle. A second division in the vesicle yields eight nuclei in total. According to Wisdom (1977) the vesicle nuclei may either dissolve or coalesce leaving one to two nuclei in the infection hypha. Allen (1923) also noted that the mature appressorium of P. g. tritici contains four nuclei and that the infection hypha contains two. The infection structures induced in vitro are morphologically and cytologically similar to those induced on the host plant (Maheshwari et al. 1967b).

Staples et al. (1984b) reported that the synthesis of nuclear DNA in U. fabae sporelings coincides with the onset of mitosis in response to the stimulus for the initiation of differentiation. The replication of DNA begins between the second and fourth hour following germination. It is not known

how external stimuli activate nuclear DNA polymerase (Yaniv and Staples 1974). In the absence of the stimulus to differentiate the uredosporeling nuclei are in G1 (i.e. period preceding DNA replication) (Staples et al. 1984b). Mitochondrial DNA synthesis occurs during germination but is not detected following the appearance of the appressoria (Staples 1974).

Uredospore nuclei are known to exist in two principal but dissimilar forms. Between divisions the interphase nucleus is described as "expanded". The expanded nucleus is composed of two parts, the "ectosphere", containing all the chromatin and the "endosphere", containing the nucleolus. As the process of division begins, the nucleus extrudes the endosphere into the cytoplasm, contracts and rounds up to form the "unexpanded" or dividing nucleus (Savile 1939). Craigie (1959) and Wisdom (1977) observed both expanded and unexpanded nuclei in P. helianthi and P. g. tritici, respectively.

2.2.2 Protein Metabolism

The changes in proteins and nucleic acids accompanying germination of uredospores and differentiation of the germtube into structures has inspired much research (Staples 1968, Dunkle et al. 1969, Kim et al. 1982a, Huang and Staples 1982, Shaw et al. 1985, Wanner et al. 1985).

Rust uredospores germinate without the appreciable increase in protein that occurs in most saprophytes, such as

Fusarium (Cochrane et al. 1971) and Aspergillus niger (Staples et al. 1962). Even so, fully functional ribosomes are present in bean and wheat rust uredospores and their capacity increases three-fold during spore hydration, prior to germination (Staples et al. 1968). Data from studies using metabolic inhibitors of RNA (actinomycin-D) and protein (puromycin) synthesis indicate that new protein synthesis is essential for differentiation. The presence of actinomycin-D during the heat shock prevents the initiation of infection structures. The presence of puromycin allows germination and appressorial formation to occur but prevents further infection structure development (Dunkle et al. 1969). The protein required for germination appears to be stored within the dormant uredospore. The mRNA responsible for directing the complete development of infection structures is synthesized at the time of heat shock. Essential protein synthesis is then programmed by the new mRNA during elaboration of the infection structures.

Although puromycin and actinomycin-D failed to prevent germination it would be erroneous to conclude that RNA and protein synthesis does not occur during the early stages of development. Uredospores contain a complete system for protein synthesis (Yaniv and Staples 1974). Germination studies on the incorporation of [^{14}C]-labelled amino acids, -glucose, and -valeric acid into protein of wheat stem rust (Reisener 1967) and bean rust (Staples and Bedigan 1967), and the appearance

of new isozymes of glucose dehydrogenase, cytochrome oxidase, and acid phosphatase (Staples and Stahmann 1964) are indicative of protein synthesis. Although there is an apparent synthesis of protein, germination is not accompanied by net protein synthesis; total free and bound amino acids remain unchanged or decrease (Staples et al. 1962).

Heat shock promotes the synthesis of new types of RNA and protein (Kim et al. 1982a, Huang and Staples 1982, Shaw et al. 1985). Although the heat shock response has been reported for a wide range of organisms its functional significance is unknown. Heat shock proteins (HSP) may serve to protect the cell against environmental stress (Ashburner 1982). Shaw et al. (1985) have identified seven HSP in Melampsora lini uredospore germlings.

Kim et al. (1982a) reported on changes in detergent-soluble polypeptides from uredospores of P. g. tritici. Dormant uredospores were found to contain more than 270 distinct polypeptides, several of which varied among physiologic races (Howes et al. 1982). After 2 and 6 hours of germination the concentrations of at least five polypeptides decreased considerably. At 2 hours, a transient concentration increase was noted for two polypeptides, 4 hours later these concentrations had decreased again to that found in dormant uredospores. The total protein extracted from six-hour-old germlings was 86 percent of the total protein extracted from dormant spores.

Differentiation was accompanied by a decrease in at least five polypeptides and an increase in four others. Two new polypeptides appeared in differentiated sporelings, their molecular weights were approximately 30.0 and 20.0 kD. Differentiated sporelings were found to contain only 48 percent of the total protein extracted from dormant uredospores (Kim et al. 1982a). Wanner et al. (1985) reported the synthesis of differentiation-specific proteins (DSP) at a time corresponding to the appearance of the substomatal vesicle.

Several possibilities exist for the observed decline in the total amount of extractable protein and certain polypeptides: Pulse-chase experiments show that polypeptides are continually turned over, existing protein is broken down into its substituent amino acids, and the amino acids are either utilized for resynthesis or lost into the medium (see Kim et al. 1982a). It is also possible that proteins bind to and form an insoluble complex with glucan and mannan residues in the cell wall of the germtube (Kim et al. 1982a). Glucan-protein and mannan-protein complexes have been detected in yeasts (Ballou 1974) and it is possible that analogous complexes occur in the germtube walls of P. g. tritici. Finally, the decline may be due to a considerable loss of metabolites and enzymes into the medium during germination and cell wall degeneration.

Pfesofsky-Vig and Brambl (1985) report that the

appearance of HSP and the depression of general translation activity is typical immediately following a temperature shift. The normal pattern of synthesis begins soon after the cells are returned to the normal temperature.

2.3 Nutritional Requirements of Rust Fungi

2.3.1 Physiology of the Host-Parasite Complex

Prior to considering the nutritional requirements of rust fungi *in vitro* it is necessary to review host-parasite relations in susceptible host tissue. The pustules of rust fungi act as foci for the accumulation of host metabolites. A compatible infection increases the rate of transpiration and respiration, decreases photosynthetic activity, alters the direction of normal phloem transport (Scott 1972) and stimulates the leakage of electrolytes from the host. These responses by the host significantly improve the availability of nutrients to the developing fungus.

With respect to structural and physiological changes associated with rust infected tissues, Bushnell (1984) recognized the juvenile and the autolytic host response. During the juvenile response most plant growth hormones increase. It is unclear however whether the observed increase is caused by pathogen or host-produced hormones. There is evidence that plant derived hormones do not play an active role in a compatible host-pathogen relationship (Levin 1985).

The increase in cytokinin activity reported by Sziraki et al. (1976) appeared to delay leaf senescence by maintaining protein synthesis (Stodart 1981). The free concentration of indole acetic acid (IAA) in plants increased two and a half fold at the infection site, 6 to 14 hr after inoculation with wheat stem rust pathogen (Artemeko et al. 1980). It is likely that IAA acts with cytokinin to control the metabolic state of the host cell.

The host nucleus and nucleolus generally enlarge during rust infection and the synthesis of nucleolar and extranucleolar RNA synthesis is enhanced (Manocha and Shaw 1966, Whitney et al. 1962, Bhattacharya et al. 1965). However, the total amount of host RNA declines which indicates that the newly synthesized RNA is rapidly degraded and turned over. Chakravorty and co-workers (1974) found that catabolic RNAse activity peaks at about 6 days at levels two to five times those of uninfected leaves.

The juvenile response is not associated with significant qualitative or quantitative changes in host proteins. Host proteins changed less in compatible host-parasite combinations than in incompatible combinations (von Broembsen and Hadwiger 1972). New isozymes have been detected on polyacrylamide gels and in most cases they appear to be of fungal origin (Johnson et al. 1968, Staples 1965).

Amino acids, amides, and carbohydrates accumulate rapidly at the infected site. During the early juvenile host response

these metabolites are synthesized locally from photosynthates and ammonia, and are also translocated from distant sites on the plant. During the later autolytic stage the local degradation of protein provides a rich source of free amino acids and amides (see Bushnell 1985).

The ratio of soluble to insoluble nitrogen increased at infections of stem rust on Little Club wheat (Gassner and Franke 1938). On a fresh weight basis bound amino acids increased almost twofold whereas free amino acids increased fourfold (Shaw and Colotelo 1961). Large increases in the amounts of free glutamine, Ÿ-aminobutyric acid (ABA), threonine and several basic and aromatic acids occurred as early as two days post-inoculation. Several investigators have reported differential increases in the amounts of asparagine, arginine, phenylalanine, leucine or isoleucine, valine (Farkas and Kiraly 1961, Shaw and Colotelo 1961) and tryptophan (Kim and Rohringer 1969).

The movement of host metabolites into parasitic mycelium has been followed with radiotracers. Labelled sucrose is inverted to glucose and fructose and readily absorbed by the rust hypha. Glucose, glutamate, alanine, glycine, lysine and arginine are taken up; the first four compounds are metabolized by the fungus. Some amino acids (e.g. serine and alanine) are absorbed from the host by the fungus more readily than others (e.g. glutamine, glutamate and aspartate). Amino acids are leached from the uredosporeling during germination

and their movement into host tissues has been reported (Jones 1966, Daly et al. 1967).

The infection of the host by a rust pathogen invariably causes an accumulation of free glutamine (Shaw and Colotelo 1961). This amide, which may be derived either by an endergonic reaction from glutamate or from proteolysis is readily translocated throughout the plant. Glutamine can serve as a source of bulk nitrogen for axenic culture (Maclean 1982) and as a precursor for the synthesis of fungal chitin (Farkas and Kiraly 1961). It is likely that glutamine plays a central role in the metabolism of rust infected leaves.

2.3.2 Axenic Culture and Metabolism

Nutritional Requirements

The nutritional requirements of P. g. tritici in axenic culture include inorganic salts, a source of carbohydrate, reduced nitrogen and reduced organic sulphur. Carbohydrate requirements are relatively nonspecific (Coffey and Shaw 1972). Nitrogen may be supplied as an inorganic ammonium salt, or as one of a wide range of organic compounds, such as an amino acid. Some amino acids are better sources of nitrogen than others. In the presence of cysteine the initiation of saprophytic growth was poor on alanine, improved on aspartate and greatest on glutamate (Coffey and Allen 1973). Sulphur must be provided in reduced form such as cysteine, cystine, the tripeptide glutathione, or methionine.

Wheat stem rust requires a higher concentration of cysteine than methionine for optimal growth (Howes and Scott 1973). The amount and the combination of sulphur containing amino acids is critical and may vary between races of rust (Singh and Sethi 1982). The nutrient requirements appear less exacting once the rust fungus becomes established on axenic medium.

Synthetic Capacity

Germinating stem rust uredospores slowly absorb, metabolize and synthesize a wide range of compounds. Uredospores germinated on a medium containing [^{14}C]-sucrose synthesize at least eleven [^{14}C]-labelled amino acids within two hours (Kasting et al. 1959). Among the first amino compounds to be synthesized were glutamine, glutamate, and aspartate (Reisener et al. 1961). Glucose is taken up readily and the carbon appears in endogenous pools of free glucose, amino acids and phosphate esters of trehalose and glucose (Manners et al. 1982). Reisener et al. (1961) demonstrated that 42 percent of the label from valerate-1-[C^{14}] was incorporated into the spore as carbohydrate; organic, fatty and amino acids; amides and peptides. Glutamine, glutamate and ABA had the highest specific activity of the free amino compounds.

Rust uredospores possess all the enzymes required for glucose catabolism and terminal oxidation (Shaw 1964), the

pentose phosphate pathway, citric acid cycle, and lipid metabolism (Caltrider and Gottlieb 1962).

Sulphur Metabolism

Most rust fungi studied so far are heterotrophic for reduced, organic sulphur when grown on chemically defined medium. Although rust fungi synthesize sulphur amino acids readily from [^{35}S]-sulphide only a limited synthesis of labelled protein can be detected (Howes and Scott 1973). More than 70 percent of the label incorporated into sulphur amino acids was lost into the medium as cysteine, S-methylcysteine, glutathione and cysteinylglycine. Labeled methionine however, appeared in mycelial protein and only negligible amounts were lost to the filtrate. P. g. tritici was unable to reduce inorganic sulphate for sulphur-amino-acid synthesis. These results suggest that a metabolic block exists in the pathway of inorganic sulphur metabolism. Howes and Scott (1973) propose that rust fungi are unable to reduce 3'-phosphoadenosine-5'-phosphosulphate to thiosulphate or thiosulphate to sulphide. It is interesting to note that sulphate reduction occurs in prokaryotes, eukaryotic algae, most fungi (Scott 1972) and all higher plants so far rested (Schiff and Hodson 1973).

Metabolite Leakage

In axenic culture the germtube and mycelium leak

metabolic intermediates. Staples and Wynn (1965), and Tulloch (1962) mention possible losses of free amino acids, sulphur-containing compounds and glycine-containing peptides to the medium but offer no quantitative data. Jones and Snow (1965) reported that [^{35}S]-labelled uredospores of P. coronata avenae lost a range of amino compounds including ethionine, ABA, methionine sulphoxide and four sulphur-containing unknowns during 12 h of germination. The amino acids detected in the culture filtrate (basal medium) of P. g. tritici after nine days were glutamate, glutamine, glycine, alanine, lysine, arginine, serine, leucine and isoleucine, phenylalanine, valine, threonine, asparagine, proline, cysteine, and methionine (Howes and Scott 1972). A considerable amount of cysteine and glycine-containing peptides, such as cysteinylglycine and glutathione accumulated in the medium. Very little methionine and homocysteine was detected (Howes and Scott 1973). From these studies it was evident that the loss of sulphur-containing compounds is selective and they are not lost as rapidly nor to the same extent as other free amino acids. ABA, Y-glutamylglutamate (Howes and Scott 1972), carbohydrate, protein, such as ribonuclease (Chakravorty and Shaw 1974) and the germination inhibitor, methyl ferulate in the cis or trans form are also leached into the medium.

Mutual stimulation during sporeling development is demonstrated by the positive effect of increasing inoculum density (Kuhl et al. 1971), and the success of "nurse culture"

techniques, "conditioned agar" (Scott 1976) and coculture experiments (Hartley and Williams 1971a, 1971b). A minimal or unbalanced medium in axenic culture would result in a net loss of metabolites, thus imposing an excessive drain on metabolism and depleting internal metabolite pools, which in turn would result in lesser growth rates (Maclean 1982). A poor approximation of amino acid balances may also result in detrimental effects, such as methionine toxicity (Howes and Scott 1972) and the selective leakage of amino acids from the mycelium (Maclean 1982).

Endogenous Free Amino Acids

Interest in the free amino acid content of rust uredospores has developed in conjunction with studies relating to self-inhibition of germination (Wilson 1958), the potential of differentiating races of rust on the basis of characteristic amino acid content (McKillican 1960), and the effects of storage on assimilative and synthetic capabilites (Wynn et al. 1966). McKillican (1960) reported race 56 as unique in that the dormant uredospore lacked glutamic acid and contained large amounts of aspartic acid and ã-alanine. Free amino acids represented approximately 0.5 percent of the original spore weight. Using the same race Stefayne and Bromfield (1965) found that the major ninhydrin-positive compounds were glutamine, glutathione, glutamic acid and ammonia. Approximately 1.2 percent of the spore weight was

composed of free amino acids. In later studies by Wynn et al. (1966) it was found that glutamic acid made up over half of the total amino acids. Other major amino acids were alanine, aspartic acid, serine, and cystine. Since no attempt was made to recover asparagine and glutamine intact, it was probable that the levels of aspartic acid and glutamic acid included these amides. Stefayne and Bromfield (1965) noted earlier that the uredospores contain seven times the amount of glutamic acid as glutamine. Analyses of the amino acid composition of wheat rust uredospores vary widely due to sampling and analysis procedures.

Accordingly, I have examined the changes in the amino acid composition of the free pool within germinating and differentiating sporelings of P. graminis tritici at 8 and 20 h following imbibition. The results are preliminary to providing information for further studies of nitrogen metabolism and nutrition in the wheat stem rust fungus.

3. EXPERIMENTAL METHODS

3.1 Production and Collection of Spores

The wheat stem rust uredospores, (*Puccinia graminis* Pers. f.sp. *tritici* Eriks and E. Henn., race C17), employed in this study were obtained through the courtesy of Dr. D. Samborski (Agricultural Research Station, Winnipeg) and were increased on *Tritium aestivum* L., c.v. Little Club.

Plants were grown in a growth chamber with a 16 h photoperiod of 9684 lux and temperatures of 25^{0}C light, 18^{0}C dark. After eight days of growth the plants were inoculated by lightly spreading a water-based paste of talc and uredospores (3:1) over the leaf blade. The plants were then well-misted with distilled water, covered with a plastic bag and placed in a dark incubator at 18^{0}C for 24 h before they were returned to the growth chamber. Within six days the leaves showed signs of "flecking", and six days following the sori opened. Five days later the uredospores were collected by gently shaking the infected leaves into a clean test tube.

The freshly harvested spores were used immediately for the amino acid analyses. In all other experiments the spores were stored in gelatin capsules at 4^{0}C for up to, but not exceeding 24 h. Germination was generally between 90 and 100 percent.

3.2 Spore Germination

Depending on the experiment, germination was carried out on either a mixed calcium and potassium phosphate (Ca-K) buffer (Table I) or MPG medium (Table II). To deplete the endogenous self-inhibitor 8 mg of uredospores were dispersed uniformly over the surface of 3 ml of Ca-K buffer in an 8-cm petri dish using an inoculation loop. After 5 min, 5 loops (5-mm diameter) of uredospores were transferred to 2 ml of the germination medium in the inner chamber of a 50-ml Conway Diffusion dish. The final spore dose was approximately 300 mm^{-2}. The outer well contained 4 ml of 1.5×10^{-4} M n-nonyl alcohol (Table I). Dishes were covered with a glass plate, sealed with vaseline and incubated in the dark at $19^{0}C$.

3.3 Spore Germination with the Induction to Differentiate

Inoculated dishes were incubated in the dark at $19^{0}C$ for 2 h, transferred to a hot water bath set at $29.5^{0}C$ for 1.5 h, then returned to $19^{0}C$. Infection structure development was complete within 20 h.

3.4 Criteria for the Assessment of Sporeling Development

A spore was considered germinated when the germtube length was equal to, or exceeded the spore diameter. Terminal

Table I: The composition and preparation of the Ca-K-PO_4 buffer and the volatile germination stimulant, n-nonyl alcohol (nonanol).

Ca-K-PO_4 Buffer	1.5 x 10^{-4}M Nonanol
$Ca(H_2PO_4)_2$ x H_2O 5 mg dissolve in 100 ml H_2O KH_2PO_4 272 mg dissolve in 100 ml H_2O add 39 ml to above K_2HPO_4 348 mg dissolve in 100 ml H_2O add to above until pH 6.8 (61 ml) Total volume 200 ml	Stock solution: dilute 1:4 prior to use N-nonyl alcohol 70 ul (9.94M) H_2O 1000 ml mix with stir bar at high speed until alcohol droplets dispersed
Inner well of Conway cell	Outer well of Conway cell

Table II: The composition and preparation of the differentiation medium MPG.

$Ca(H_2PO_4)_2 \times H_2O$	0.025 g
KH_2PO_4	0.449 g
K_2HPO_4	1.145 g
Peptone	5 g
D-Glucose	30 g
volume made to with glass distilled H_2O	1000 ml

The KH_2PO_4 and $Ca(H_2PO_4) \times H_2O$ were dissolved in 200 ml distilled water. K_2HPO_4 was added until pH 6.8 was reached. Peptone and glucose were added and the pH was adjusted as necessary with phosphoric acid or KOH. The medium was sterilized by autoclaving.

swellings were counted as appressoria only after a septum delimiting the appressorium from the germtube was clearly visible. The infection peg was recognized as a small outgrowth from the mature appressorium. Substomatal vesicles were recognized after the terminal end of the infection peg had expanded to a size equal to half that of the appressorium. As the vesicles matured they flattened out forming two lobes on either side. An infection hypha was counted when one lobe of the vesicle extended beyond the length of the other.

3.5 Staining and Counts

Sporelings were transferred from each dish to a glass slide, stained with trypan blue in lactophenol (Table III), covered with a coverslip and sealed with nailpolish. The slides were semi-permanent and may be stored for up to one year. Counts of 100 spores were made per slide and the component infection structures were individually assessed. Differentiation was expressed as a percentage of the total spores.

3.6 Temperature Range Trials

Temperature requirements for appressorium formation and the total differentiation of uredospore germlings was investigated by applying a heat shock of constant temperature

Table III: Lactophenol-Trypan Blue; a mounting and staining medium for semipermanent mounts of fungi (Boedijn, 1965).

Phenol	20	g
Lactic acid	20	g
Glycerol	40	g
Distilled water	20	ml
Trypan Blue	0.5%	

(ranging from 26 to 32^{0}C for 1.5 h) 2 h following germination at 19^{0}C. The temperature range was tested over four sub-experiments, each of which utilized a different spore lot. The heat shock applied during the temperature range trials was administered by placing the Conway dishes on a temperature regulated brass plate. Warm water (35^{0}C) from a hot water bath was circulated through copper tubing under one end of the brass plate while the ambient temperature cooled the other end. The temperature gradient (25 to 35^{0}C) thus generated was allowed to stabilize for at least 2 h prior to the experiment. Following 2 h germination at 19^{0}C the inoculated dishes were transferred to the brass plate and set at various intervals along the temperature gradient. After 1.5 h the temperature of the Ca-K buffer in each dish was measured by an electronic thermometer and a thermocouple. The dishes were then returned to 19^{0}C for 14 h. Observations were made after a total of 17.5 h of incubation.

Since the germination and differentiation capacity of spore batches differed slightly, the data within each sub-experiment were adjusted to allow for comparison between experiments. The maximum differentiation value within each sub-experiment was represented as "100"; all other values were calculated as a percentage of the maximum. The optimum differentiation temperature varied between experiments and ranged from 28.2 to 30.7^{0}C. Therefore, the optimum temperatures were assigned a value of "0", all remaining

temperatures were calculated as units deviating from this optimum.

3.7 Essential Protein Synthesis: Puromycin

Uredospores were inoculated onto the Ca-K buffer and induced to differentiate under conditions previously described. A protein synthesis inhibitor, Puromycin dihydrochloride (100 ug/ml) (Sigma Co.), was dissolved in the buffer and added to, or removed from (using a 10-ml syringe), the inner well of the Conway dish at the times specified in Table IV. After each medium change, the sporelings were washed three times with fresh medium. Essential protein synthesis was measured by assessing the extent of uredosporeling differentiation after 15 h.

In all treatments designed to examine essential protein synthesis the uredospores were killed and stained in lactophenol-trypan blue upon completion of the experiment. Each treatment had three replicate dishes, two sample counts were taken from each replicate, and 100 spores were counted per sample. The treatments were compared to the control for each morphogenic group by one-factor analysis of variance. The complete experiment was repeated twice.

Table IV: The timing of essential protein synthesis: Experimental design. The uredosporelings were germinated at 19^0C for 2 h, exposed to a heat shock for 1.5 h, then returned to 19^0C for 12 h.

Treatment	Stages of germination and differentiation (h) at which puromycin is added to the medium (x)					
	0-2	2-3.5	3.5-6	6-8	8-10	10-15
(control)	-	-	-	-	-	-
2	-	-	-	-	-	x
3	-	-	-	-	x	x
4	-	-	-	x	x	x
5	-	-	x	x	x	x
6	x	x	x	x	x	x
7	-	-	x	-	-	-
8	-	-	-	x	-	-
9	-	-	-	-	x	-
10	-	-	x	x	-	-
11	-	-	-	x	x	-

3.8 Nuclear Staining: DAPI

Uredospores were germinated on Ca-K buffer for 4 h in the dark at 19^{0}C. The germlings were transferred to a clean slide using an inoculation loop and allowed to dry. A drop of DAPI (4',6-diamidino-2-phenylindole), 1 ug/ml glass-distilled water, was placed directly on the sample, a coverslip was applied and the edges were sealed with nailpolish. The slide was observed within 20 min by fluorescent microscopy.

The instrument used was a Zeiss universal microscope equipped with a 100 W mercury lamp to deliver excitation light by epifluorescent mode, a Zeiss UG1 exciter filter with a passband from ca 300 to 400 mu, BG38 to absorb light and protect sample from heat, a barrier filter with a cut off at 410 nm, and a Zeiss Neofluor 40 power objective. Transmitted light and fluorescence photographs were made with Fujicolor 400 ASA daylight film.

The results obtained from unfixed material were unacceptable in that DAPI stained the germtube wall as well as the nuclei. Several fixation schedules were tested in order to optimize stain specificity (Table V). All fixed samples were placed on a glass slide and stained in a drop of 1 ug/ml DAPI.

All tissues that were fixed in alcohol-containing fixatives were washed in alcohol of the same concentration as that present in the fixative. After washing the tissue was

Table V: A description of the fixation schedules tested.

1. Formalin-acetic acid-alcohol (FAA)*

Ethyl alcohol (50%)	90 ml
Glacial acetic acid	5 ml
Formalin	5 ml

The material was fixed for 2, 24, and 48 h, then rehydrated through an alcohol series to water.

2. Chrom-acetic solution (Johansen, 1940)

Aqueous chromic acid (10%)	2.5 ml
Aqueous acetic acid (10%)	5.0 ml
Distilled water	to 500 ml

The sample was fixed for 20 h then washed three times in distilled water (30 min per wash).

3. Ethanol/Acetic acid (3:1)

The material was fixed for 2, 24, and 48 h, dried on a slide, immersed in 200 mM KCL and rehydrated through a graded alcohol series.

4. Formaldehyde: 4% and 36% *

The sample was fixed for 20 h in either a 4% solution or the vapours of 36% formaldehyde, then washed 3 times in distilled water (30 min per wash).

5. Glutaraldehyde: 3% *

The sample was fixed for 1 h then washed three times in distilled water (30 min per wash).

6. Ethanol series

The material was dehydrated through an ethanol series (15%, 30%, 40%, 60%, 70%) allowing 5 min between changes. After at least 4 h in 70% the sample was rehydrated (10 min between washes) back to distilled water.

* These fixatives were both used as described and in combination with a surfactant. Two surfactants were assessed individually, 0.25% Triton X-100 and 0.01% Tween-20.

left for 30 min in each of the following: 50%, 30%, 15% ethyl alcohol, and distilled water.

The method of choice was a simple alcohol dehydration and subsequent rehydration of the sample prior to staining with DAPI. The nuclei were clearly visible and background fluorescence was minimimal in all but the very young germtubes (0 to 2 h). Nuclear fluorescence improved for 20 min and remained stable for up to 10 days when stored in the dark at 4^0C. The chemical structure of DAPI is shown in Figure 2.

3.9 Amino Acid Analysis

3.9.1 High Performance Liquid Chromatography (HPLC)

HPLC separations are carried out in high resolution columns packed with 3 to 25 m particles of uniform size distribution. The columns require the use of dedicated injectors for sample introduction, sensitive detectors, and special pumps which deliver constant flow against high pressure. HPLC methods may be used for the separation of a diverse array of compounds with molecular weights ranging between 50 and 20 million.

Two approaches are currently available for HPLC analysis of amino acids: analysis using reversed-phase separation of prederivatized amino acids, and analysis using ion-exchange methods with post-derivatized amino acids.

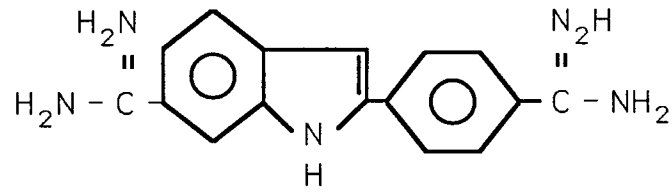


Fig. 2. DAPI (4',6-diamidino-2-phenylindole) binds specifically to AT residues of double-stranded DNA. The AT-specificity resides with both the guanidine group and the indole ring, which may bind to the purine of adenosine through base stacking (Otto and Tsou, 1985).

Reverse-Phase (RP) Bonded-Phase Chromatography

The stationary phase of a RP column is non-polar, consisting of silica gel with covalently bound hydrocarbon chains (lengths ranging from C_1 to C_{22}). The elution profile of a sample reflects the degree of hydrophobicity inherently unique to each of its components. The compound with the highest affinity for the solid-phase emerges last. Solvents from high to intermediate polarity are used as mobile phases. Thus, water is the solvent which gives the longest retention. The retention and selectivity of the column can be adjusted and optimised by the addition of water-miscible organic solvents (e.g. methanol, acetonitrile). Since the conditions of separation are mild the formation of artifacts is not a problem.

3.9.2 Reagents

Type I reagent grade water was prepared by running glass-distilled water through the Milli-Q water purification system (Millipore Co). This system combines activated carbon adsorption, mixed-bed deionization, an organics-scavenging cartridge (Organex-Q), and 0.22 m sterilizing membrane filtration (Millipak Filter Unit).

Methanol and acetonitrile were HPLC-grade (BDH Chemical Co). All remaining components of the buffer system were ACS grade. Amino acid standards (hydrolyzate mix, No. 20088), trifluoroacetic acid (TFA), triethylamine (TEA), and the

derivatizing agent phenylisothiocyanate (PITC) (No. 26922) were obtained from the Pierce Chemical Company.

3.9.3 Instrument

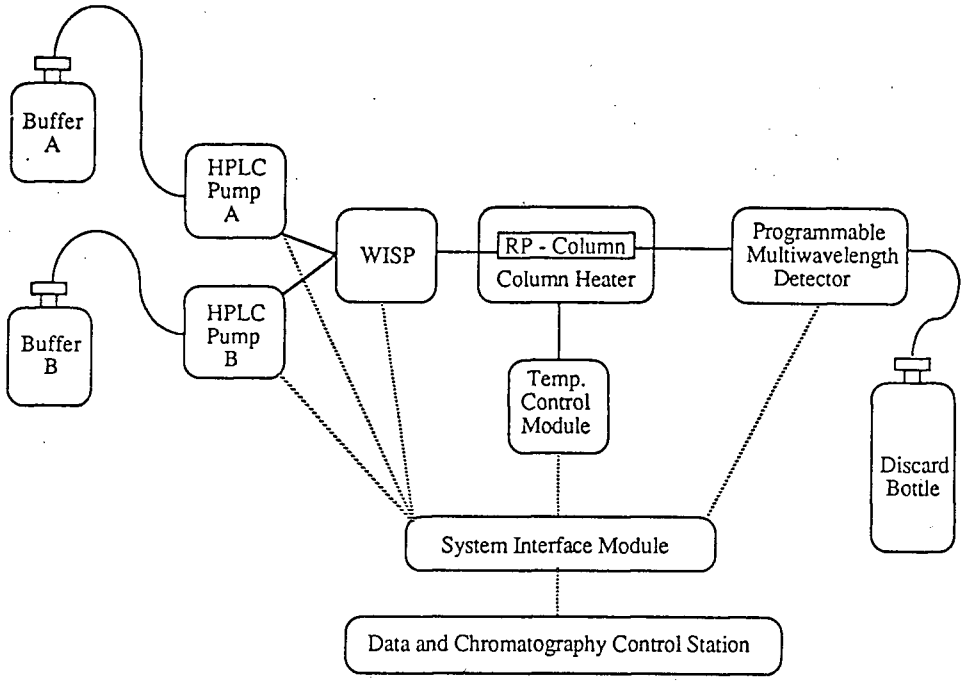
The equipment used was designed by Waters and consisted of two Model 510 pumps, a Model 710B WISP, a System Interface Module, an 840 Data and Chromatography Control Station, a Temperature Control Module, and a 490 Programmable Multiwavelength Detector (Figure 3). The stationary phase for RP was a Waters C_{18} Pico-Tag column for protein hydrolyzates (3.9 mm x 15 cm). RP-HPLC was performed at 41^{0}C.

When not in use the column and pumps were washed with water to remove salts and then stored in acetonitrile.

3.9.4 Sample Collection and Preparation

The procedures for amino acid analysis necessitated strict control of all possible sources of contamination. Bacterial and fungal contamination were monitored by suspending uredospores in sterile water and plating serial dilutions onto nutrient agar.

Glassware: Glassware was first washed in soap (Alconox) and water, rinsed with tap water, oven dried and then soaked in an acid bath (NoChromix, Chemonics Sci.) for at least 1 h. The glass was then rinsed three times with glass distilled water, rinsed once in Milli-Q water and dried at 60^{0}C for several



Component (Waters)	Function
Pump A (Model 510)	delivery of elution buffer A
Pump B (Model 510)	delivery of elution buffer B
WISP (Model 710B)	automated sample injection
System Interface Module	links seperate components with Control Station
840 Data and Chromatography Control Station	controls conditions for HPLC, stores and analyzes data
Amino Acid Analysis Column	reverse-phase separations
Temperature Control Module	regulation of column and coil temperature
Programmable Multiwavelength Detector (490)	detection of PITC-derivatives

Figure 3. Waters HPLC amino acid analysis system configuration for RP-analysis of amino acids with post-column ultraviolet detection.

hours.

Buffer: Ca-K buffer was prepared with Milli-Q water in acid-washed glassware, sterilized through a 0.2 m Millipore filtration unit and stored at 4^0C until required.

Spore Germination: Procedures earlier described for inducing germination and differentiation were carried out using autoclaved, acid-washed Conway dishes and sterile buffers.

Ungerminated, non-differentiated, and differentiated uredospores and their leachates were analyzed for free amino acid content. The undifferentiated and differentiated sporelings were harvested after 8 and 20 h. Each treatment consisted of six dishes, which were later combined to be analyzed as one sample. The 20 h experiment was repeated three times and the 8 h experiment twice. The extent of differentiation was monitored for each treatment using the method described in Staining and Counts (page 30).

Wash: Freshly harvested spores (50 mg) were shaken in 10 ml of buffer and 0.01% Tween-20 for 5 min, the suspension was centrifuged and the supernatant transferred to an acid-washed vessel. The above procedure was repeated three times yielding a wash volume of 30 ml which was then freeze-dried and taken up in 1 ml of Milli-Q water. The washed spores were ground and extracted as described earlier.

Spores: The spores were collected, rinsed with Milli-Q water, placed in an acid-washed mortar, ground with acid-washed sand and HPLC-grade 80% ethanol, and then transferred to a centrifuge tube and stored at 20^0C for 20 h. The spore extract was centrifuged at high speed for 10 min and the deproteinized supernatent decanted into an acid-washed vessel. The ethanol was evaporated to 1 ml under a stream of nitrogen gas and transferred to a 10-ml test tube. The dry weight of 50 mg spores was determined by drying the spores for 16 h at 100^0C.

Leachate: The leachate, together with the water used to rinse the spores, was collected from the 6 dishes with a 10-ml syringe, filtered (0.2 m Millipore) into a 150 ml acid washed pyrex vessel, shell frozen in liquid N_2 and freeze-dried. The residue was taken up in 2 ml of Milli-Q water and transferred to a 10-ml test tube.

3.9.5 Sample Clean-up

A C_{18} Sep-Pak cartridge (designed by Waters) was used to remove lipid, pigment, residual protein, and other hydrophobic materials from all samples. The sample preparation scheme is given in Figure 4. Following clean-up all samples, including a standard amino acid mixture, were dried down in a SpeedVac concentrator (Savant Instruments) without heat.

1. Activate a Sep-Pak C_{18} cartridge with 2-10-ml volumes of methanol.

2. Wash with 2-10 ml of 0.1% trifluoroacetic acid (TFA) in Milli-Q water.

3. Wash with 10 ml of 0.1% TFA in water and methanol (80:20).

4. Mix 1 ml sample with 2 ml 0.1% TFA in water and methanol (70:30).

5. Pass the sample through the Sep-Pak.

6. Discard the first 1 ml of effluent and collect the next 2 ml fraction which contains all the amino acids.

Figure 4. Sample preparation scheme for crude amino acid samples using Waters Sep-Pak C_{18} cartridges.

3.9.6 HPLC of Amino Acids as Phenylthiocarbamoyl Derivatives

Buffer System

Eluent A: Sodium acetate trihydrate, 19.0 gm (140 mM) was dissolved in 1000 ml of Milli-Q water and 5% TEA. The pH was adjusted to 6.4 with glacial acetic acid. The solution was vacuum filtered and combined with 25 ml acetonitrile.

Eluent B: Contained 60% acetonitrile in Milli-Q water. Degassed by sonication for 5 min.

Pre-column Derivatization

Derivatization Solution: An ethanol, TEA and Milli-Q water (7:1:1) solution was combined with 10% PITC.

Sample Diluent: A phosphate buffer was prepared (710 mg Na_2HPO_4 per 1000 ml titrated to pH 7.4 with 10% phosphoric acid), combined with 50 ml acetonitrile, and filtered.

Procedure: The dried sample and standard were derivatized with 20 l of the derivatizing solution for 30 min at room temperature. The solvents were removed under vacuum with the SpeedVac centrifuge. The sample and the standards were reconstituted with 25 and 500 l of sample diluent, respectively. The amino acids were derivatized in order to improve the detection sensitivity as well as overcome the inherent polarity of the free amino acids. PITC-amino acids

have a broad UV spectrum with a maximum absorbance near 269 nm.

PITC allows the quantitative derivatization of both primary and secondary amino acids. The reaction scheme for amino acids by pre-column derivatization with PITC is outlined in Figure 5.

Sample Preparation

Prior to being loaded on the column each sample was filtered (MSI, Cameo dispensable nylon filters, 3 mm membrane, 0.45 m pore size) and collected into 0.5 ml Eppendorf micro test tubes by centrifugation for 5 min in a microcentrifuge (Eppendorf Model 5413). The tubes were then placed on a spring in 1.5 ml glass vials (Pierce Chemical Co.), covered with a membrane, sealed with a cap, and placed on the autosampler tray. The pump table was programmed to generate the appropriate buffer gradient system and flow-rate (Table VI).

Chromatography

Four 1 of standard were injected per run (200 pM), the first injection generated the "junk" chromatograph and was discarded, the second injection produced the chromatograph used to calibrate the column and subsequent runs. The sample injection volume was 8 1 and the injection-to-injection cycle time was 30 min. After four injections and separations of

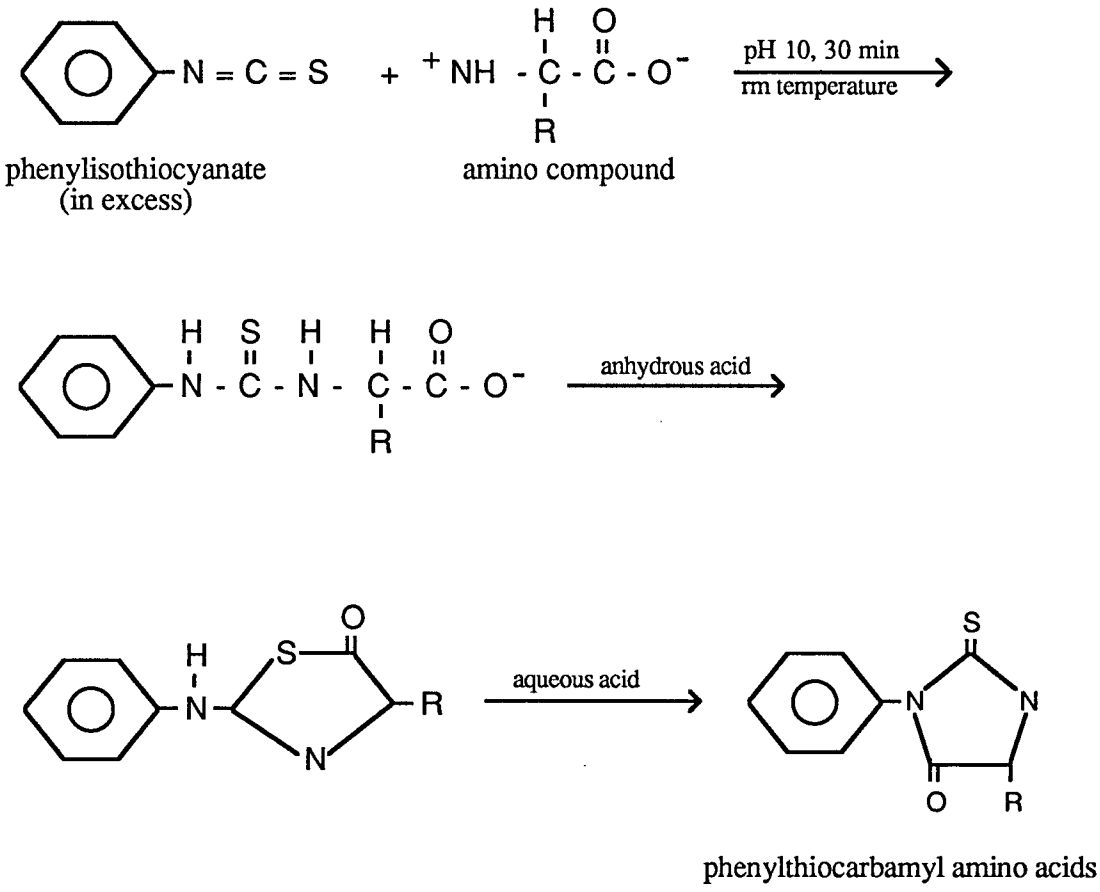


Fig. 5. Reaction scheme of amino compound by pre-column derivatization with PITC.

Table VI: Pump method; the buffer gradient system and flow-rate designed to effectively contribute to the separation of amino compounds within a physiological sample.

Time	Flow	Curve	%A	%B
Initial conditions	1.0	*	95	5
10.00	1.0	5	55	45
10.50	1.0	6	0	100
11.50	1.0	6	0	100
12.00	1.5	6	0	100
12.50	1.5	6	100	0
20.00	1.5	6	100	0
20.50	1.0	6	100	0
30.00	0.1	11	100	0

sample the column was recalibrated with the standard.

The relationship between a specific amino acid and a known concentration was calculated in terms of a response factor (RF). The RF is an adjustment for compounds that do not respond equally in the detector (RF= peak area/amino acid concentration). The sample chromatograms contained a number of peaks. Most of the retention times corresponded with those of identified amino acids, the remaining peaks were classified as "unknown". The RF values determined during the calibration run were used to calibrate the amount of a specific amino acid within the sample. Standard solutions of glutamine and glutathione were prepared, and 200 pM of the standard was injected onto the column. Glutamine formed a peak between serine and glycine and glutathione eluted with ammonia.

Three controls were run: The Ca-K buffer contained a large sloping peak (eluting at approximately 1 to 2.2 min). The baseline was otherwise flat with negligible peaks. Acid washed sand ground with 80% ethanol generated a chromatogram with a flat baseline interrupted by two sharp peaks. The first peak eluted at 9.65 min, shortly after leucine, and the second followed phenylalanine at 11.1 min. The blank derivative contained no interfering amino compounds.

4. RESULTS

4.1 Uredospore Germination and Differentiation "In Vitro"

In the presence of 1.5 x 10^{-4} M n-nonyl alcohol, 90 to 100% of the uredospores germinated within 2 h following seeding on Ca-K buffer (Fig. 6).

Contrary to earlier studies in which 95% differentiation was obtained (Maheshwari et al., 1967; Dunkle et al., 1969), the proportion of germtubes forming complete infection structures on Ca-K buffer did not exceed 60%. The timing of infection structure formation in response to a 1.5 h heat shock (30.5^{0}C) on Ca-K buffer is shown in Fig. 7. Germtube growth was arrested shortly after exposure to the elevated temperature. The hyphal tip began to swell after 3.5 h and within 5 h the appressorium was mature (i.e. a cross wall delimited the appressorium from the germtube). The infection peg formed between 5 and 6 h and at 8 h began to extend to produce the substomatal vesicle. The development of the vesicle was usually completed within 10 h by the formation of a septum between it and the appressorium. After 10 h the infection hypha began to develop on the distal end of the vesicle. The cytoplasm within the germtube and the appressorium began to clear at 8 and 9 h, respectively. Infection structure formation was complete within 15 to 20 h.

Initially the induction of differentiation by heat shock

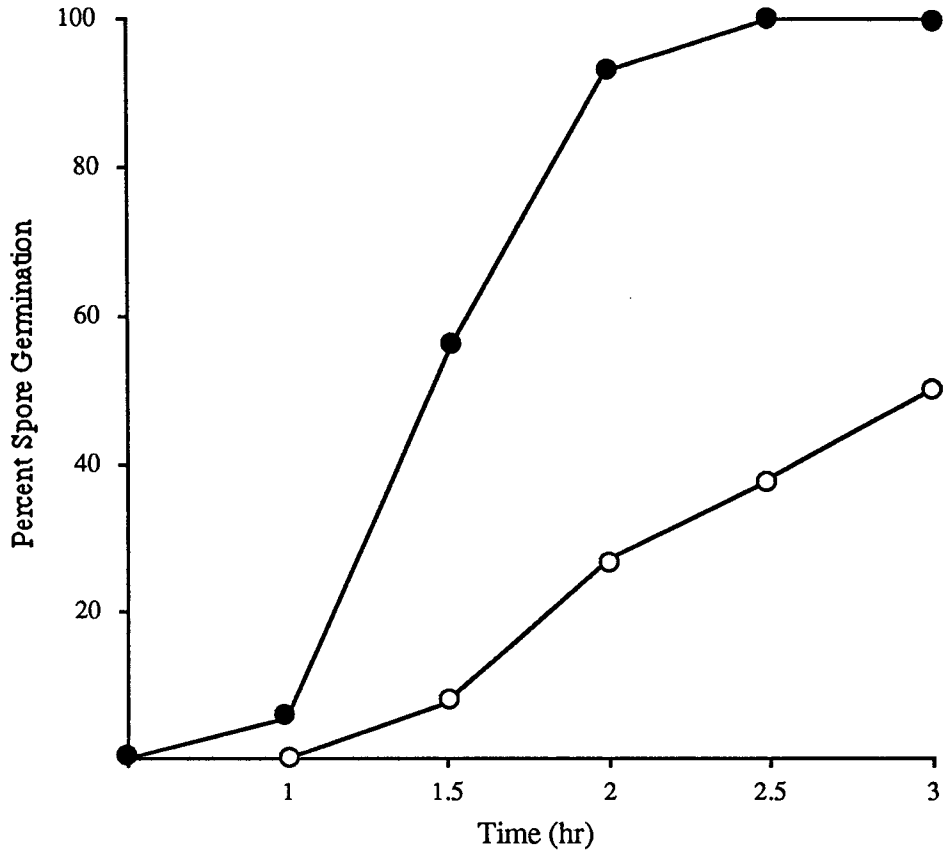


Fig. 6. The percent germination of uredospores on Ca-K buffer in both the presence (●) and absence (O) of 1.5 x 10^{-4} M n-nonyl alcohol.

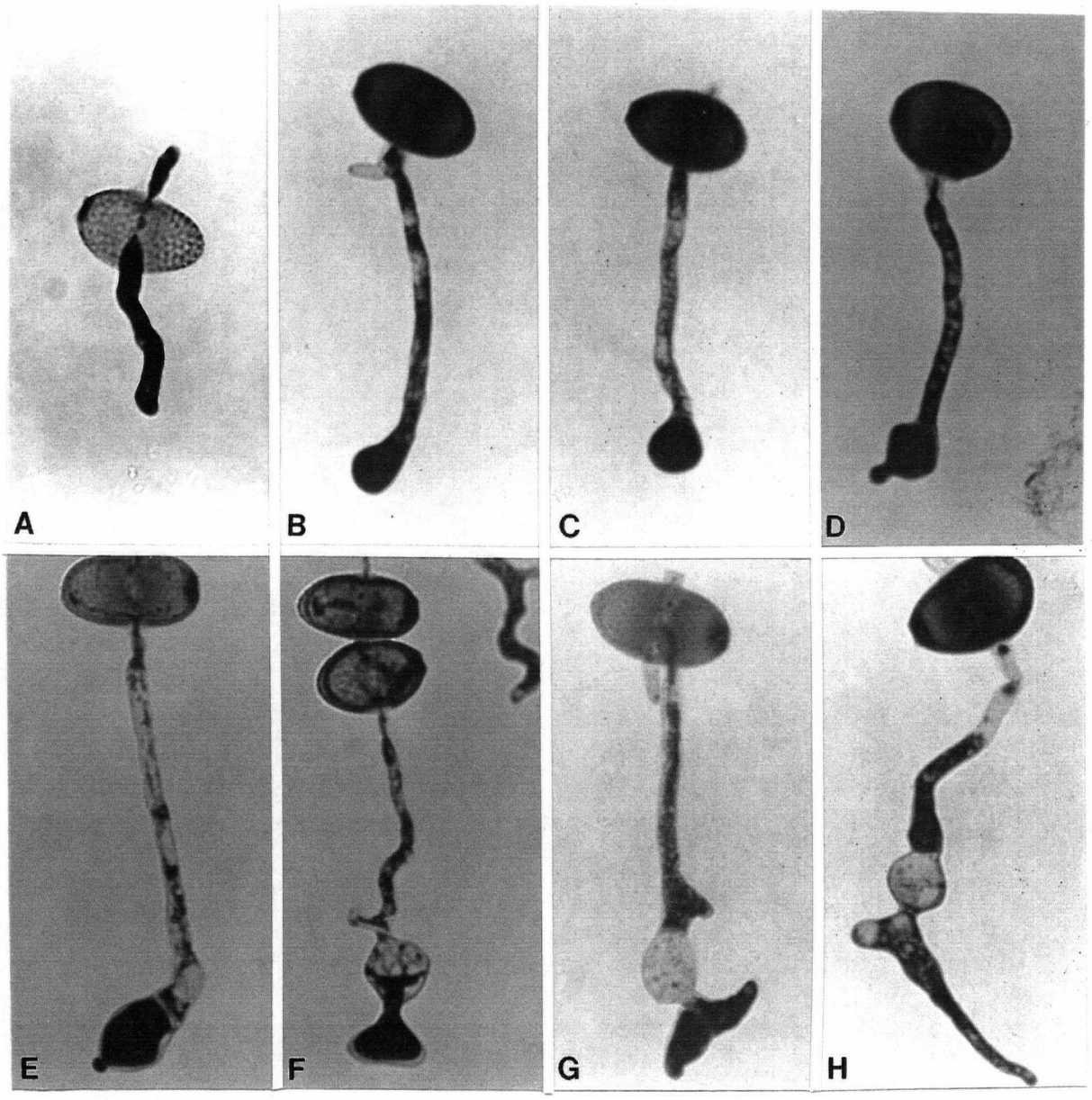


Fig. 7. Timing of sporeling morphogenesis. (A) A sporeling 1 h following germination. (B) A sporeling 3.5 h following germination, the appressorium initial has formed. (C) After 5 h a septum was visible and the appressorium was considered mature. (D) Development of the infection peg was complete 6 h following germination. (E) After 9 h the cytoplasm began to clear within the germtube. (F) The substomatal vesicle began to expand after 8 h. (G) The cytoplasm migrated from the appressorium into the developing substomatal vesicle and a second septum become apparent (10 h). (H) The infection hypha was apparent after 15 h. A differentiated sporeling after 20 h. (592 X).

as described by Maheshwari et al. (1967) yielded poor results (< 5% differentiation). By using a spore density of 300 per mm^2 and controlling the temperature of the heat shock by placing the Conway dishes in a water bath, it was possible to attain up to 60% differentiation. All further attempts to improve the percent differentiation; such as, changing the timing and duration of the heat shock were unsuccessful.

4.2 Temperature Range Trials

The temperature required to induce the maximum proportion of germtubes to differentiate into infection structures was found to lie within a narrow range. The optimum temperature appeared to be characteristic of a particular spore lot and ranged from 28^0 to 30^0C. Incubation at lower than 27.5^0C or higher than 30.5^0C considerably reduced the amount of appressorium formation (Fig. 8) and total differentiation (Fig. 9). The upper and lower temperature limits for all spore lots tested were estimated as 30.5^0 and 27.5^0C, respectively. Heat treatment was completely ineffective when the temperature was raised 1^0 above the upper limit or dropped 2^0 below the lower limit (Figs. 8 and 9).

Although the incubation temperature where the maximum total differentiation was achieved varied between spore lots, the sensitivity of the sporelings to temperature changes was similar (Figures 10 and 11). Variations 1^0 above or below the

Fig. 8. Temperature requirements for appressorium formation from uredospore germtubes. The spores were germinated at 19^{0}C (2 h), then placed at constant temperatures (ranging from 26 to 33^{0}C) for 1.5 h, and replaced to 19^{0}C. The optimum temperature (><) is the temperature at which the maximum total differentiation was attained for each spore lot.

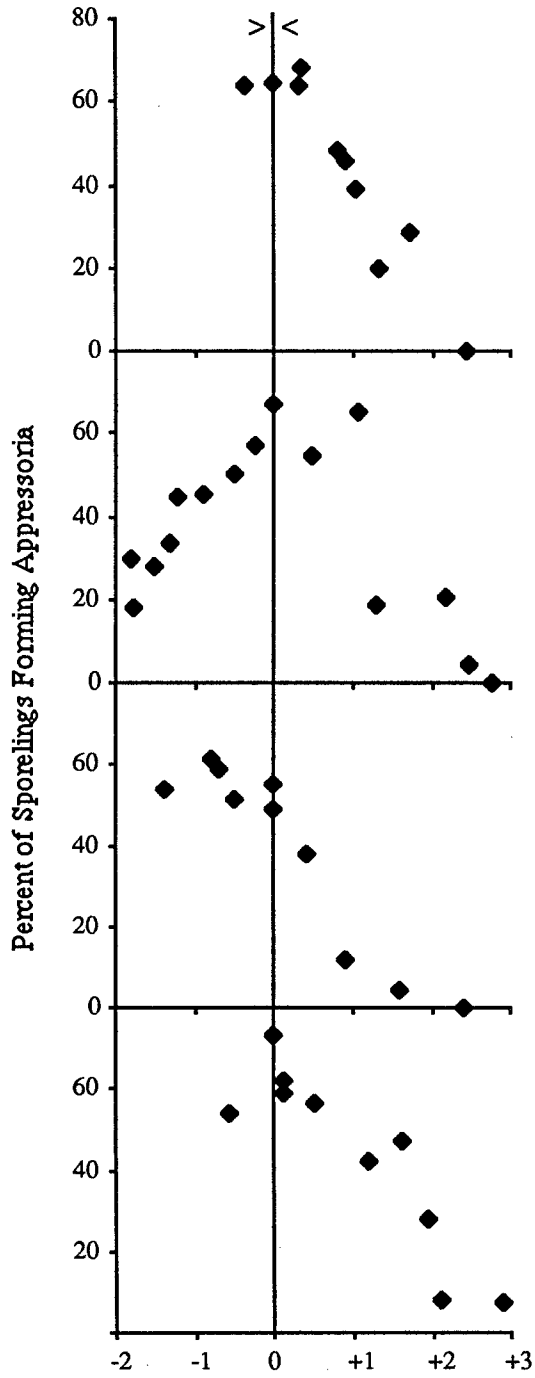


Fig. 8A. Spores collected July 22, 1986. Optimal differentiation temperature (> <): 29.3 °C.

Fig. 8B. Spores collected July 29, 1986. Optimal differentiation temperature (> <): 28.2 °C.

Fig. 8C. Spores collected August 6, 1986. Optimal differentiation temperature (> <): 30.7 °C.

Fig. 8D. Spores collected November 13, 1986. Optimal differentiation temperature (> <): 28.8 °C.

Fig. 9. Temperature requirements for the complete differentiation of P. graminis tritici uredospore germlings. The upper and lower limits for total differentiation were estimated to be 27.5 and 30.5^{0}C, respectively. Germination conditions were described in Fig. 8.

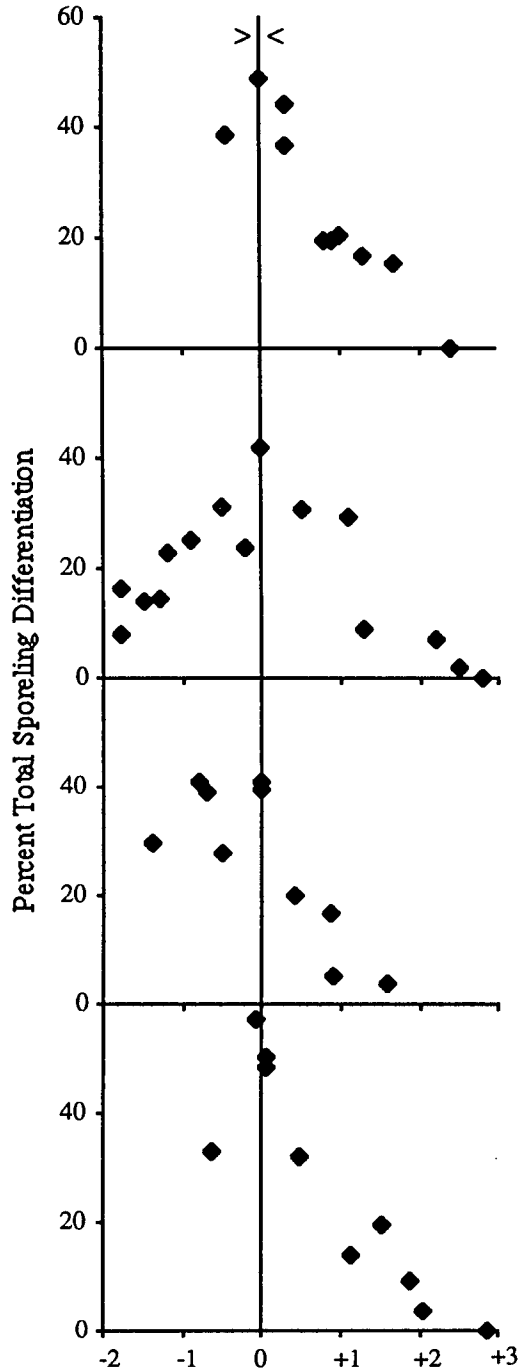


Fig. 9A. Spores collected July 22, 1986. Optimal differentiation temperature (> <): 29.3 °C.

Fig. 9B. Spores collected July 29, 1986. Optimal differentiation temperature (> <): 28.2 °C.

Fig. 9C. Spores collected August 6, 1986. Optimal differentiation temperature (> <): 30.7 °C.

Fig. 9D. Spores collected November 13, 1986. Optimal differentiation temperature (> <): 28.8 °C.

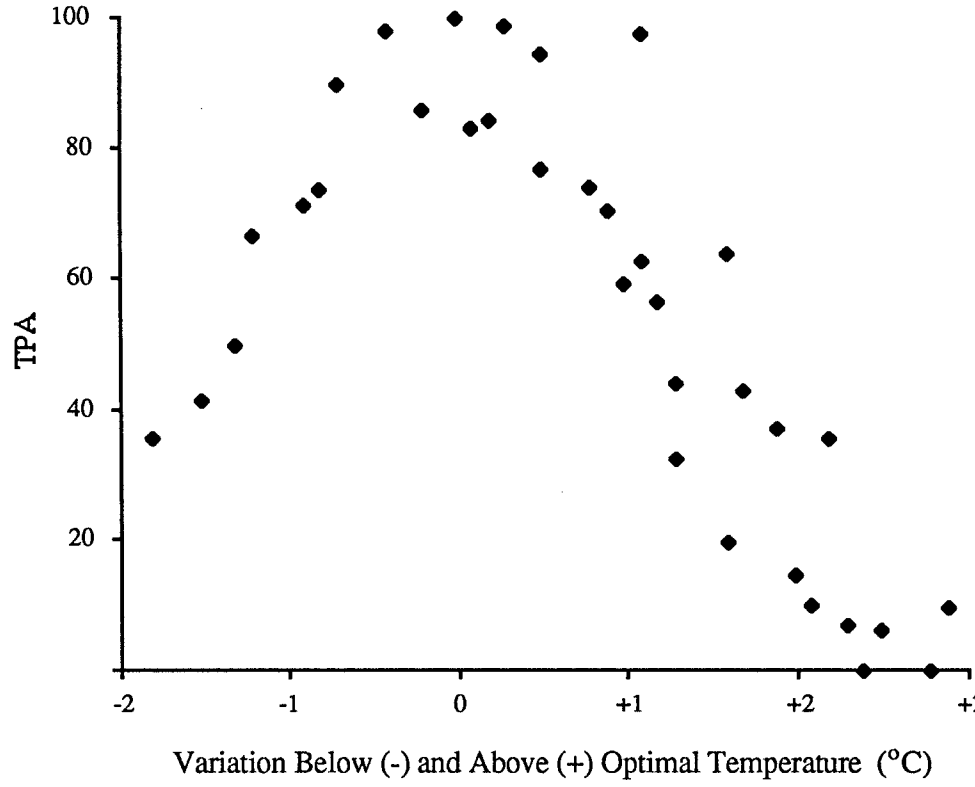


Fig. 10. The relationship between heat shock temperature and the transformed percent of appressoria formed (TPA).

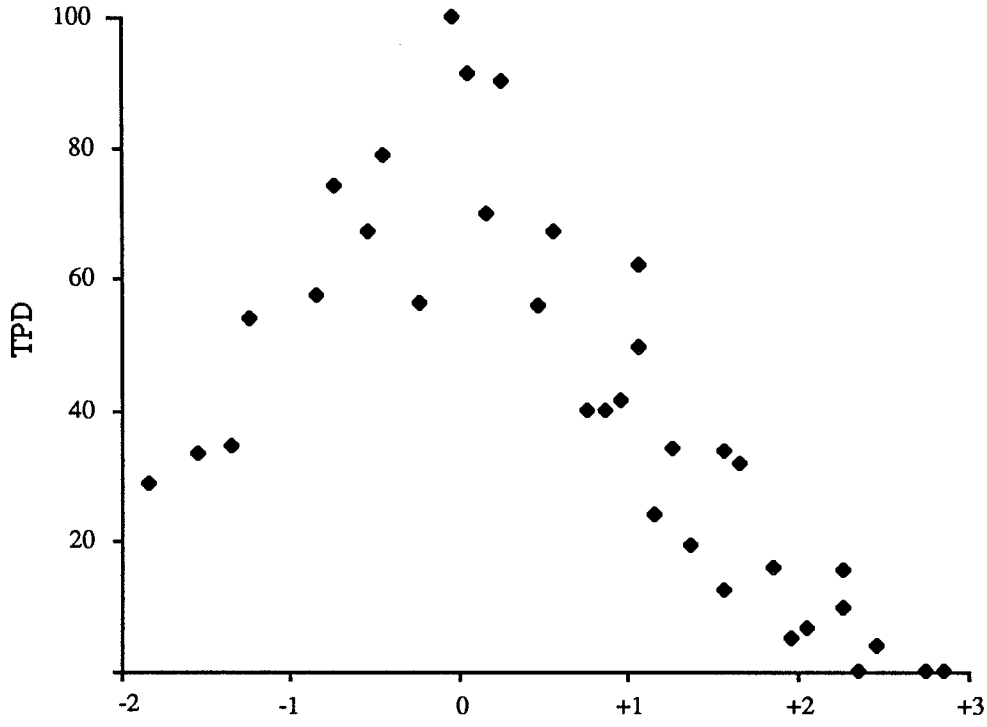


Fig. 11. The relationship between heat shock temperature and the transformed percent of total sporeling differentiation (TPD).

optimum temperature resulted in greater than 40% reduction in the number of sporelings forming infection structures. The formation of appressoria was less sensitive to deviations from the optimum temperature (Fig. 10) than the formation of a complete set of infection structures (Fig. 11).

4.3 The Influence of Nutrients on Sporeling Differentiation

Preliminary experiments were undertaken to investigate the effect of nutrients (peptone and glucose), a germination stimulant (n-nonyl alcohol), and a heat shock (30^{0}C, 1.5 h), on the percentage of sporelings developing infection structures (Table VII). MPG medium (peptone, glucose, and Ca-K buffer), in conjunction with both a heat shock and n-nonyl alcohol, supported up to 77% differentiation. When the heat shock was not administered, less than 11% of the sporelings differentiated. The absence of both, n-nonyl alcohol and the heat shock resulted in the growth of long, matted germtubes that failed to form infection structures.

Although all three components of MPG influenced sporeling differentiation it was evident that their roles were not of equal importance. MPG was broken down to its constituent parts and each component was tested for its ability to promote differentiation. The buffer alone supported 66% differentiation, peptone in distilled water supported 14%, and glucose in distilled water resulted in less than 5% sporeling

Table VII: A summary of experiments investigating the influence of the components of MPG, n-nonyl alcohol, and a 29.5^{0}C (1.5 h) heat shock on the percent of germtubes forming appressoria (PAS) and the percent of sporelings forming a complete set of infection structures (PDS). The presence or absence of a treatment is indicated by '+' or '-', respectively.

MPG			n-nonyl alcohol	heat shock	PAS	PDS
peptone	glucose	Ca-K				
+	+	+	+	+	89	77
+	+	+	+	-	27	11
+	+	+	-	+	55	50
+	+	+	-	-	0	0
+	+	-	+	+	54	44
+	-	+	+	+	71	32
-	+	+	+	+	84	39
+	-	-	+	+	36	14
-	-	+	+	+	83	66
-	+	-	+	+	5	5
* -	+	+	+	+	40	32

*supplemented with tyrosine (49 mg/l), cysteine (17 mg/l), and tryptophan (15 mg/l).

differentiation. The combination of peptone and glucose increased differentiation to 32%. The maximum amount of uredosporelings forming a complete set of infection structures (77%) was achieved on the complete MPG medium. These results show clearly that the response to nutrients is non-additive.

Glucose in distilled water promoted the growth of long germtubes which failed to differentiate. The addition of cysteine, tyrosine and tryptophan to glucose and Ca-K buffer had no significant effect on differentiation.

4.4 Essential Protein Synthesis

Sporelings exposed to an inhibitor of protein synthesis before, during, and after the inductive period provide evidence for the nature and timing of protein synthesis during differentiation. It was found in the present study that the presence of puromycin (100 ppm) had a significant effect on appressorium formation and completely inhibited all further development (Table VIII). In most cases where the heat treatment arrested linear growth the germtube produced an apical swelling, which after 20 h failed to form a cross wall (Fig. 12a). The appressorium was earlier defined by Staples et al.(1983c) as a swelling of the hyphal tip that is separated from the germtube by a septum. In the absence of puromycin the appressoria were oval to roundish. In the

Table VIII: The effect of a protein inhibitor, puromycin (100 ppm), on the development of infection structures from germlings of P. graminis tritici. To induce the differentiation of infection structures a heat shock at 30°C for 1.5 h was administered 2 h after the start of germination at 19°C.

Treatment	Sporeling development (%)							
	Appressoria		Infection peg		Vesicle		Infection hypha	
	a*	b#			a	b		
------ (control)	69	100	61	100	53	100	40	100
-----x	78	114	72	117	60	114	27	67
----xx	73	107	69	112	50	96	1	3
---xxx	72	105	58	94	17	31	0	0
--xxxx	75	109	54	88	0	0	0	0
xxxxxx	17	25	2	3	0	0	0	0
--x---	78	114	71	115	49	93	21	53
---x--	76	111	67	109	43	81	12	30
----x-	78	113	72	118	46	88	11	27
--xx--	72	105	57	93	27	52	3	7
---xx-	73	106	55	90	27	51	2	5

Puromycin was present (x) and absent from (-) the germination medium (Ca-K buffer). (------) corresponds to time intervals; 0-2 h, 2-3.5 h, 3.5-6 h, 6-8 h, 8-10 h, and 10-15 h. *development is expressed as a mean percent of the total spores counted. #development is expressed as a percent of the control. underlined values are significantly different from the control (95% confidence - Fisher LSD).

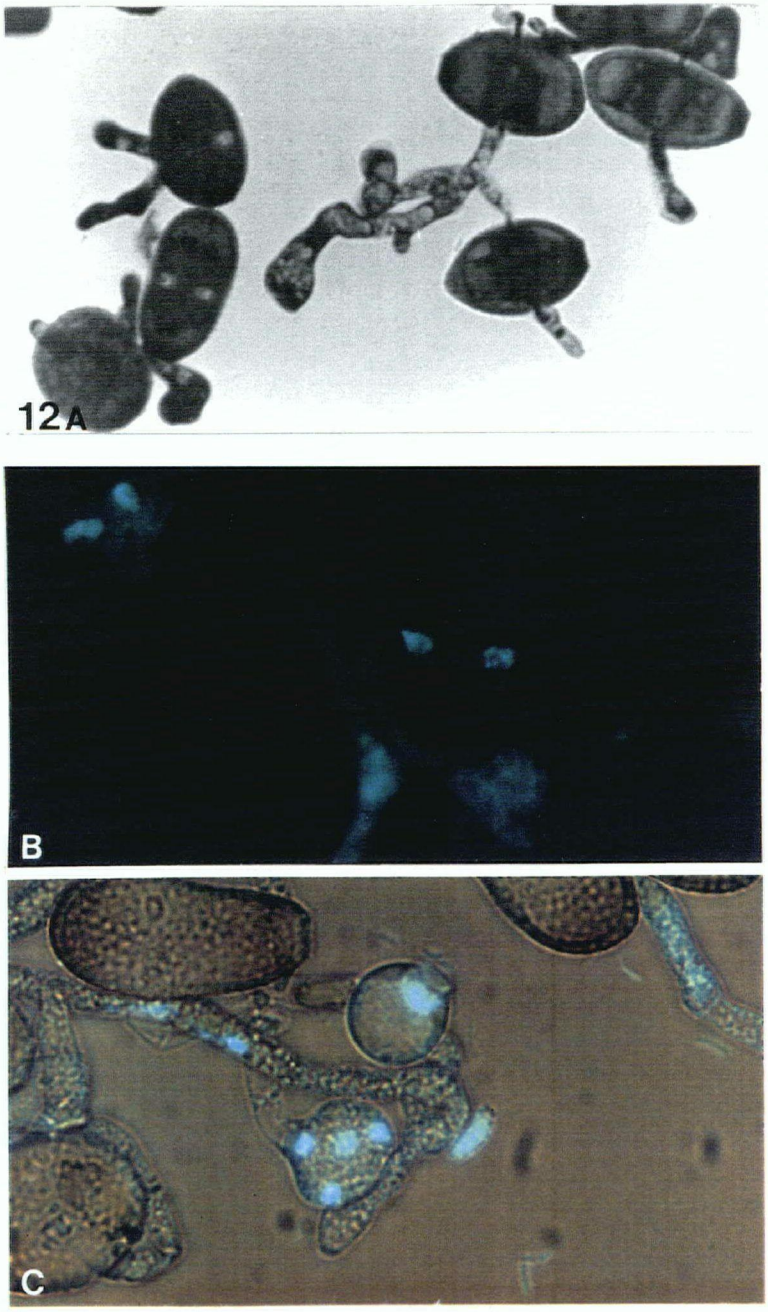


Fig. 12. The presence of a protein inhibitor (puromycin) in the germination medium significantly reduced the number of germtubes forming appressoria. (A) The appressoria were predominantly irregular in form (bright field illumination, 592x). (B) Generally the nuclei within the germtube did not divide but remained expanded from 0 to 15 h (fluorescent micrograph, 1040x). (C) In cases where a septum was formed the nuclei divided to give rise to four daughter-nuclei. The pairing of daughter nuclei was not observed (double illumination with U.V. and highly attenuated visible light, 1040x).

presence of the inhibitor however, their shape was predominantly irregular (Fig. 12a).

The results found by Dunkle et al. were confirmed (with the exception of appressorium formation) by a series of preliminary experiments. To further clarify the timing of protein synthesis and its effect on the proportion of germtubes forming infection structures the sporelings were exposed to puromycin at specific times following heat treatment. Complete differentiation was prevented in those trials where puromycin was present from immediately after heat treatment to the completion of the experiment (20 h). During this time frame the development of the appressorium and the infection peg were unaffected but all further differentiation was inhibited (Tables VIII and IX). Although the sporelings appeared to recover from the effects of the inhibitor shortly after the removal of puromycin from the germination medium, the development of the infection hypha was significantly impaired in all cases.

Although the development of vesicles did not occur before 10 h, their formation appeared to be dependent on proteins synthesized 3.5 to 8 h following germination. The formation of the infection hypha was dependent on the development of the vesicle; the latter always preceded the former. The proportion of vesicles developing infection hyphae was considerably reduced when puromycin was present at 3.5 to 8, 6 to 10, or 6 to 15 h and completely inhibited when the

Table IX: The effect of a protein inhibitor, puromycin (100 ppm), on the development of infection structures from germlings of P. graminis tritici. Development is expressed as a mean percent of the preceding structure.

Treatment	Sporeling development (%)					
	Infection peg		Vesicle		Infection hypha	
	a*	b#	a	b	a	b
------ (control)	90	100	86	100	76	100
-----x	92	103	84	98	44	59
----xx	94	105	73	85	2	3
---xxx	80	90	29	34	0	0
--xxxx	72	80	0	0	0	0
xxxxxx	10	11	0	0	0	0
--x---	91	101	69	81	43	57
---x--	88	98	64	74	28	37
----x-	93	104	64	75	23	31
--xx--	79	88	48	56	10	13
---xx-	76	85	49	57	7	10

Puromycin was present (x) and absent from (-) the germination medium (Ca-K buffer). (------) corresponds to time intervals; 0-2 h, 2-3.5 h, 3.5-6 h, 6-8 h, 8-10 h, and 10-15 h. *development as a mean percent of the preceding structure. #development as a mean percent of the preceding structure, expressed as a percent of the control. underlined values are significantly different from the control (95% confidence - Fisher LSD)

inhibitor was present at 3.5 to 15 h (Tables VIII and IX).

The nuclear behaviour of sporelings differentiating in the presence of puromycin was interesting. Following heat treatment the 2 nuclei migrated from the spore, into the germtube and towards the appressorial initial. In most cases the nuclei remained in the germtube, had a granular appearance, and were expanded in form (Fig. 12b). Occasionally the nuclei migrated into the developing appressorium and completed one round of mitotic division. The presence of an inhibitor of protein synthesis appeared to arrest germtube development at a specific phase of the cell cycle. The expanded form of the nuclei suggests that the cell was either in the last part of G1 or entering the S-phase. The four daughter nuclei did not move together but remained separate and expanded throughout the observation period (at least 20 h) (Fig. 12c). Nuclear elongation prior to mitotic division was not observed.

4.5 Cytology of Uredosporeling Development

4.5.1 Nuclear Staining

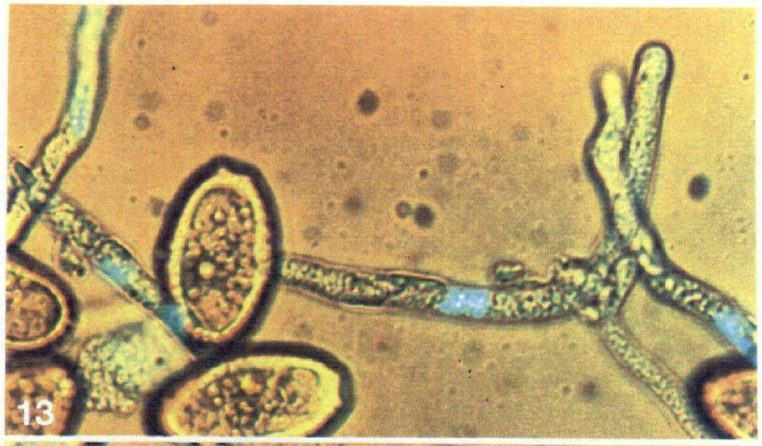
The most suitable technique tested for fixation was a graded ethanol series followed by staining with 1 ug/ml DAPI. DAPI reacted rapidly with the sample, the nuclei were clearly visible and fluoresced a bright blue. Most of the germtubes remained transparent with little to no fluorescence.

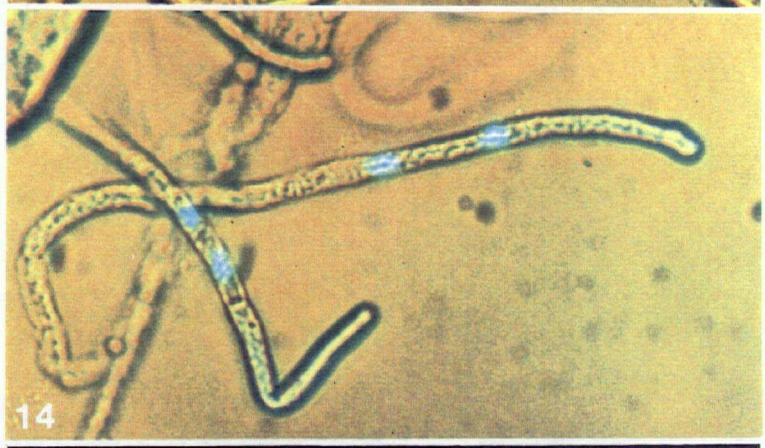
Germtubes 1.5 h old or younger fluoresced strongly making it difficult to discern the nuclei. Nuclear fluorescence improved for 20 min and then remained stable for up to 10 days when stored in the dark at 4^{0}C. In cases where the infection hypha was developing and the nuclei had migrated into the hypha a change was observed in the vesicle. The vesicle cytoplasm became speckled, the "granules" fluoresced a bright gold.

The addition of a surfactant, such as Triton X-100 or Tween-20 allowed the spores to be taken into suspension and simplified washing procedures by centrifuging the sample between changes. However, in the presence of surfactants the germtube fluoresced strongly rendering the nuclei invisible.

4.5.2 Nuclear Behaviour during Germination and Differentiation

For cytological studies the uredospores of P. graminis tritici were germinated on a Ca-K buffer. The sporelings were induced to differentiate as described earlier (page 27). Uredospore nuclei were stained with DAPI at various stages of germtube development. The resting uredospore was binucleate. Following germination the two nuclei usually migrated in tandem from the spore into the developing germtube and toward its apex (Fig. 13). These nuclei were round to oval in shape and represented the expanded or interphase nuclei described by Savile (1939). In the absence of a stimulus to differentiate





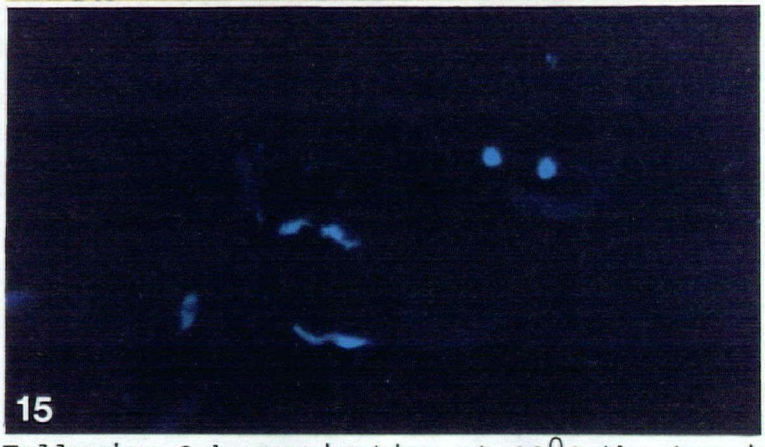
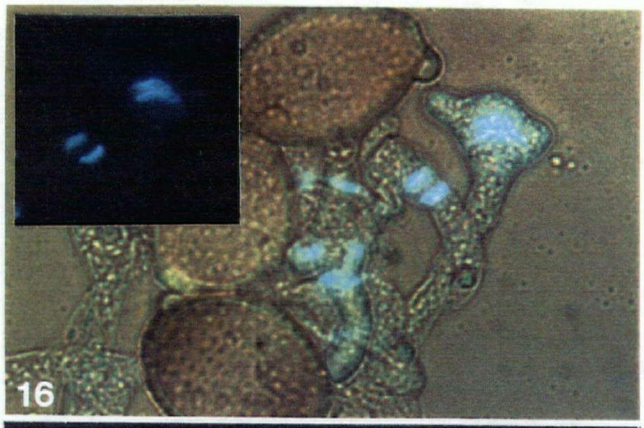
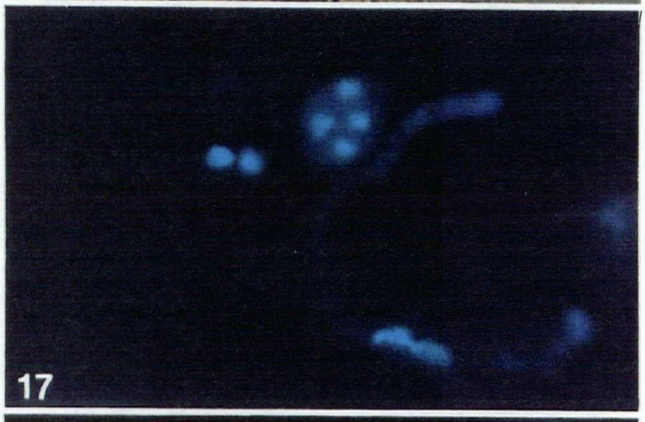


Fig. 13. Following 2 h germination at 19^{0}C the two interphase nuclei migrated from the uredospore into the elongating germtube (Double illumination with U.V. and highly attenuated visible light, 1040x).

Fig. 14. The germtube of non-shocked uredosporelings remained binucleate for up to 20 h. The nuclei usually appeared to remain in perpetual interphase (1040x).

Fig. 15. In some cases the nuclei within 20-hour-old uredosporelings were elongated in form and had a "ragged" appearance (fluorescent micrograph, 1040x).





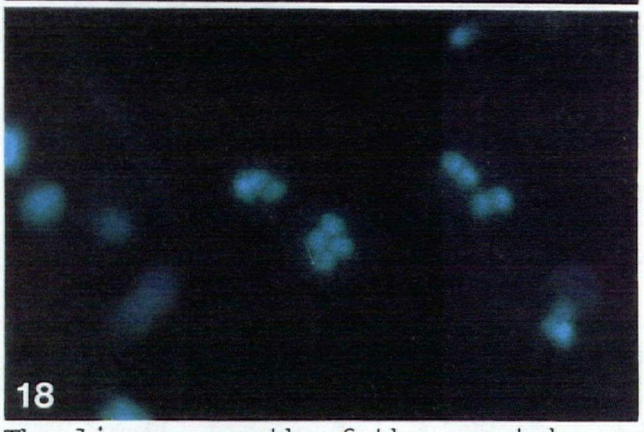


Fig. 16. The linear growth of the germtube was arrested during the 1.5 h heat shock period. Four hours following germination the two nuclei had migrated into the newly formed appressorium initial. Their elongated form suggested that these nuclei were in conjugate telophase and preparing to divide (double illumination with U.V. and highly attenuated visible light, inset: fluorescent micrograph, 1040x).

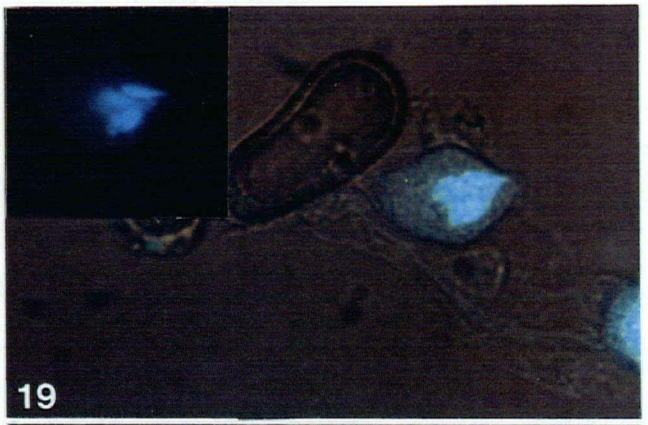
Fig. 17. The first round of mitosis usually occurred within the mature appressorium 5 and 8 h after germination (1040x).

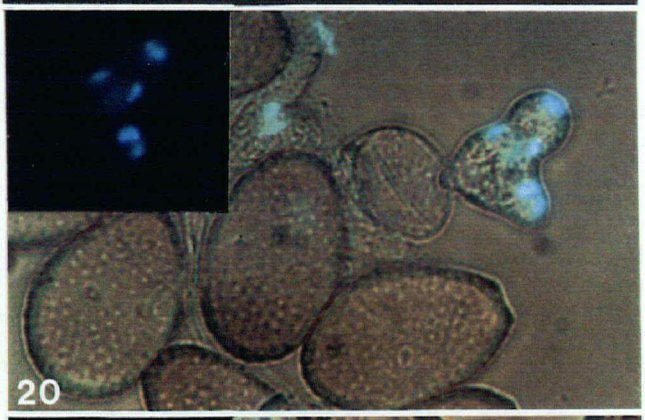
Fig. 18. The mature appressorium contained four daughter-nuclei. These nuclei moved together to form a compact tetrad (1040x.

the germtube remained binucleate and appeared to be in perpetual interphase; mitotic division was not observed in 0- to 20-hour-old sporelings (Fig. 14). In some cases the nuclei of nondifferentiated 20-hour-old sporelings had a "ragged" appearance and became elongated in form (Fig. 15).

The nuclear behaviour of the differentiating sporeling was complex; nuclear division was predictable and changes in form and migration patterns were observed regularly. Although nuclear division was readily observed the more subtle changes in nuclear form, which correspond to specific phases of the cell cycle could not be followed with certainty. During heat shock the germtube produced a bulbous appressorial initial into which the two nuclei migrated. At 4 h the nuclei were elongated to dumbbell in shape and were situated one behind the other (Fig. 16). Their appearance suggested that these nuclei were in conjugate telophase and nearing the completion of mitosis. Usually, after 4.5 h a cross wall formed and delimited the appressorium from the germtube. The first round of mitosis was usually complete within 5 to 8 h following imbibition (Fig. 17). The mature appressorium contained four nuclei arranged as a compact tetrad (Fig. 18).

At 7 h the four nuclei migrated toward the developing infection peg. Occasionally two nuclei divided in the appressorium (Fig. 19) but more commonly the second division occurred as the nuclei moved into the developing substomatal vesicle (7 h to 13 h). The nuclei migrated in closely





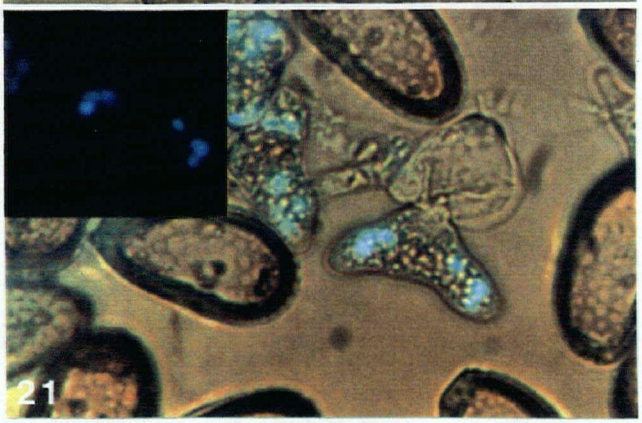


Fig. 19. Occasionally two nuclei divided within the appressorium. The six nuclei migrated in tandem towards the developing infection peg (double illumination with U.V. and highly attenuated visible light, inset: fluorescent micrograph, 1040x).

Fig. 20. Ten hours following germination the six-nucleate vesicle was delimited from the appressorium by a septum (1040x).

Fig. 21. In most cases a nuclear pair divided in the mature vesicle to yield a total of eight nuclei. These nuclei were distributed such that three to four nuclei aggregated on either side of the vesicle (1040x).

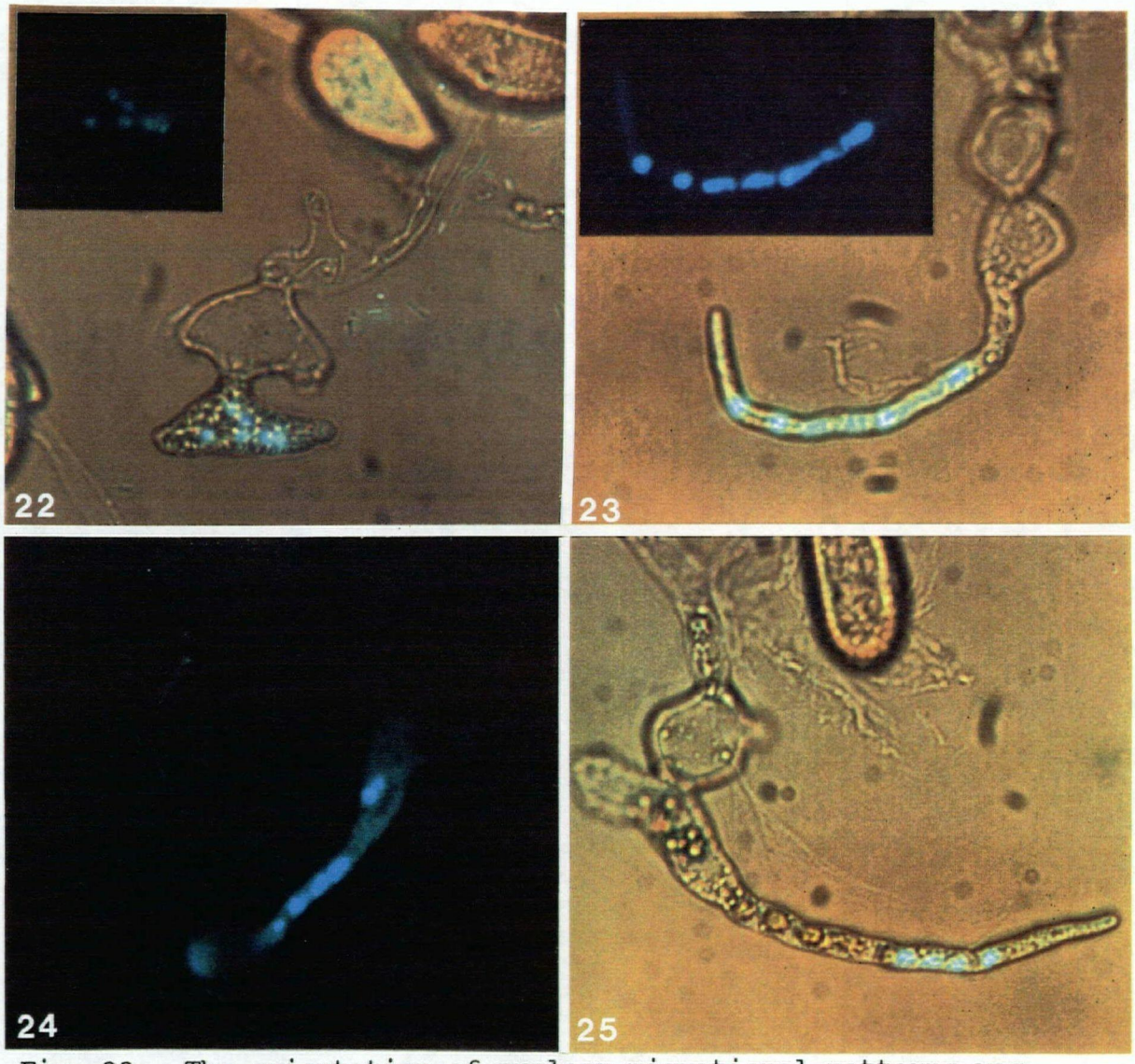


Fig. 22. The orientation of nuclear migrational patterns was found to be related to the polarity of the vesicle. Once growth of the infection hypha was initiated the nuclei moved into an arrowhead formation with the apex directed towards the developing hypha (double illumination with U.V. and highly attenuated visible light, inset: fluorescent micrograph, 1040x).

Fig. 23. The infection hypha contained eight, six, or more commonly four nuclei (1040x).

Fig. 24. In some cases a nuclear pair failed to migrate with the other nuclei and remained in the vesicle (1040x).

Fig. 25. Twenty three hours following germination the infection hypha most often contained four expanded nuclei (1040x).

associated pairs.

A cross wall delimited the six-nucleate vesicle from the appressorium within 10 h (Fig. 20). In most cases a nuclear pair divided in the mature vesicle to yield a total of eight nuclei (Fig. 21). These nuclei, which were rounder and considerably smaller than those observed in the germtube, are described as dividing or unexpanded. Nuclear division was not always synchronous, five to eight nuclei were often seen within the vesicle. Within 10 to 13 h after germination six to eight nuclei were distributed such that three to four nuclei aggregated on either side of the vesicle (Fig. 21).

The infection hypha of P. graminis tritici developed as a polar extension from the vesicle. Once growth of the infection hypha was initiated (ca 13 h) a nuclear migration pattern became apparent. The nuclei moved towards the developing hypha in the formation of an arrowhead (Fig. 22). The orientation of their migration was always relative to the polarity of the vesicle. The leading nucleus, which often fluoresced more strongly than the others, was directed towards the developing hypha. As growth ensued eight, six or four expanded nuclei migrated into the infection hypha (Fig. 23), occasionally a nuclear pair remained in the vesicle (Fig. 24). After 23 h four or fewer nuclei were observed in the hypha (Fig. 25).

4.6 Amino Acids Analysis

The standard generated 18 peaks with characteristic retention times. The separation of a PITC-amino acid standard is illustrated in Fig. 26, Table X. Good resolution was observed within 12 min. An additional 18 min was required to remove solvent peaks and reequilibrate the column between runs. Except for histidine from arginine, and glutathione from ammonia, most amino compounds were adequately separated using the RP-column . As is characteristic of RP-chromatography, the elution order was related to the increasing hydrophobic nature of the solute (i.e. the more water soluble the compound, the faster it was eluted).

4.6.1 Experimental Plan

The objective was to determine the effect of the heat shock on (A) the endogenous free amino acid pool and (B) the exogenous free amino acids leaching from the germinating uredospore. The following fractions were therefore analyzed:

(1) Buffer wash from resting uredospores.

(2) Alcohol extract from unwashed resting uredospores.

(3) Alcohol extract from eight-hour-old uredosporelings: non-shocked and heat shocked.

(4) Leachates from eight-hour-old uredosporelings: non-shocked and heat shocked.

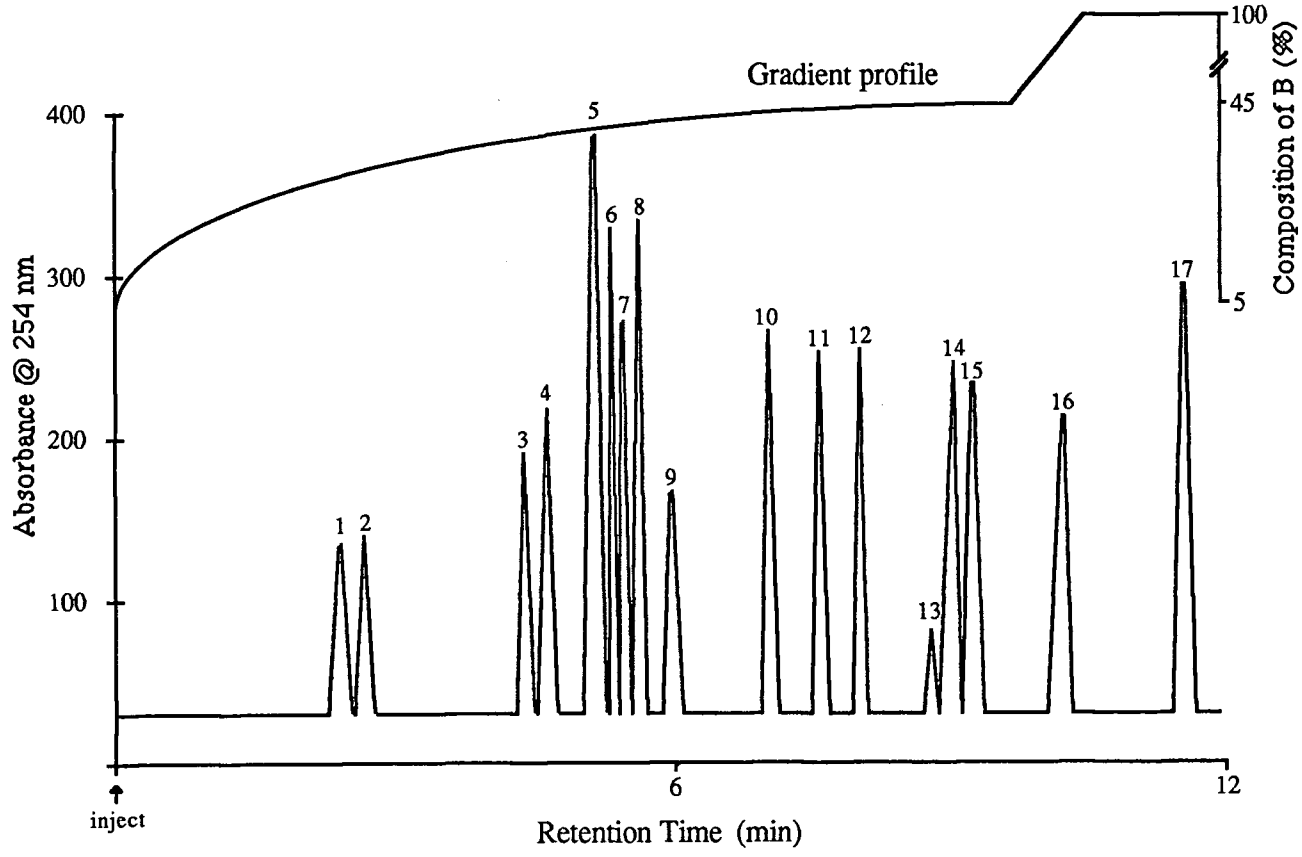


Fig. 26. Separation of amino acid standards (Pierce H). Eluent A: 140 mM sodium acetate, 5% TEA, pH 6.4; eluent B: 60% acetonitrile in water; gradient: 5% B to 45% B in 10 min on curve 5; flow-rate: 1 ml/min for 12 min, 1.5 ml/min for 8 min: column: Pico-Tag RP-column for protein hydrolyzates; detector: ultraviolet (254 nm) at 0.1 a.u.f.s. For peak identification see Table X.

Table X. PITC-amino acid peaks identified by number and retention time as separated by RP-HPLC under the conditions shown in figure 26.

Number	Amino Acid	Retention Time (min)
1	Aspartic acid	2.44
2	Glutamic acid	2.68
3	Serine	4.48
4	Glycine	4.69
5	Histidine / Arginine	5.22
6	Threonine	5.37
7	Alanine	5.51
8	Proline	5.66
9	NH_3	6.00
10	Tyrosine	7.06
11	Valine	7.61
12	Methionine	8.00
13	Cysteine	9.12
14	Isoleucine	9.30
15	Leucine	9.50
16	Phenylalanine	10.27
17	Lysine	11.62
18	Glutamine	4.53
19	Homoserine	4.96
20	Glutathione	6.00
*	Unknown peaks	--

(5) Alcohol extract from 20-hour-old uredosporelings: non-shocked and heat shocked.

(6) Leachates from 20-hour-old uredosporelings: non-shocked and heat shocked.

4.6.2 Complications

A number of problems were encountered in identifying and quantifying the amino compounds in uredospores and uredospore leachates. Representative RP-HPLC amino acid profiles from (A) the alcohol extract from non-shocked uredospores, and (B) the leachate from heat shocked uredospores, are shown in Fig. 27. Two peaks corresponding to glutamine (#18 - see Table X) and NH_3/glutathione (#9/20) are predominant in both profiles. The resolution between peaks identified as serine, glycine, and glutamine was poor. Therefore, since column selectivity could not be improved, strictly quantitative estimates of the amounts of these amino compounds per mg spores could not be obtained. Despite the overlap, peaks corresponding to serine, glycine, and glutamine were identified in most, but not all extracts. The most serious overlap occurred between glycine and glutamine and in Fig 27 glycine was completely obscured by glutamine (#18). In those runs where the separation between glycine and glutamine was achieved the glycine present was always only about 1/3 the height of the glutamine peak. The response factors (RFs) for glycine and glutamine were similar in value. In order to calculate the order of magnitude of the

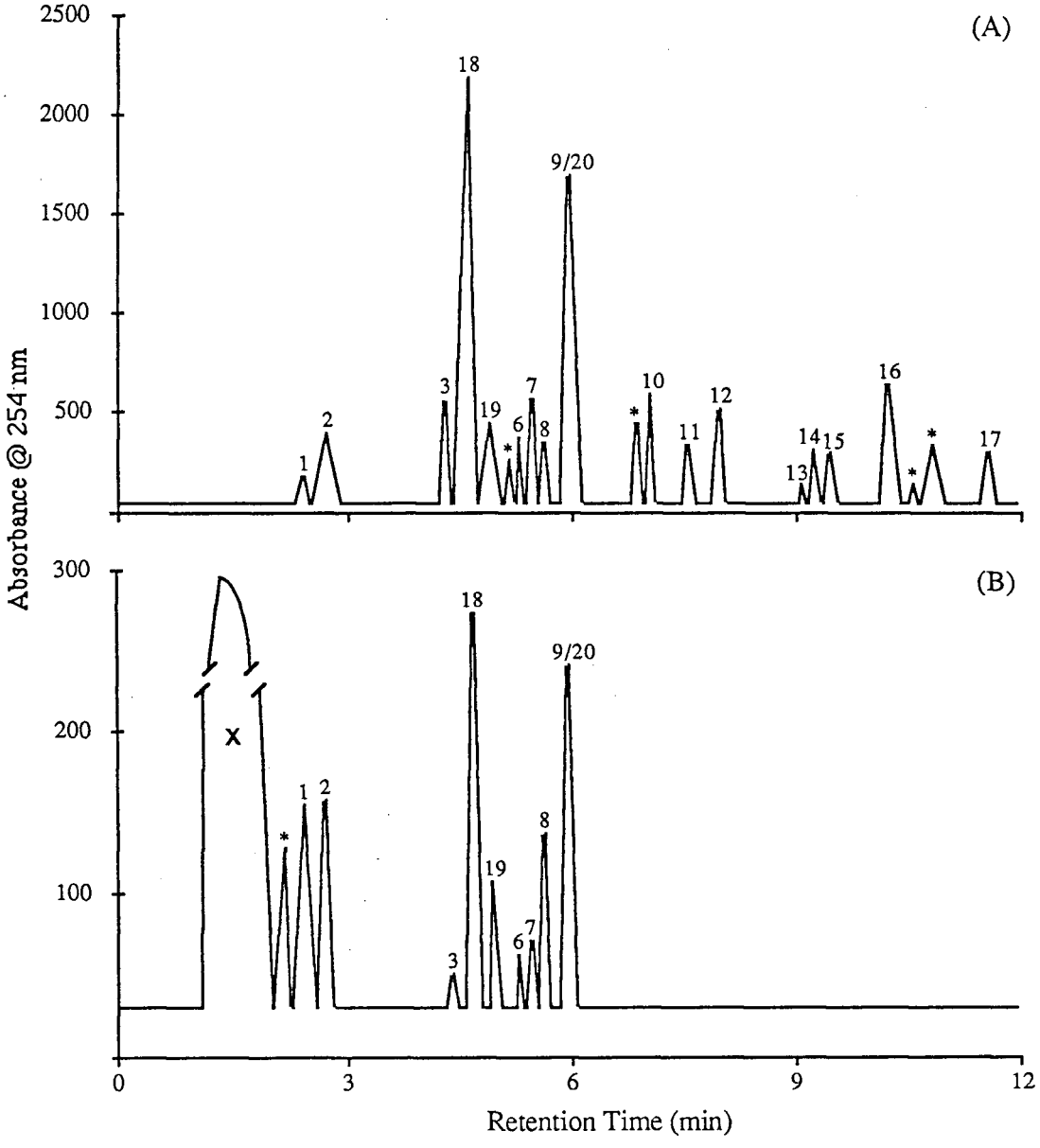


Fig. 27. Representative RP-HPLC separations of amino acids isolated from (A) non-heat shocked 20-hour-old uredosporelings and (B) the leachate of 20-hour-old heat shocked uredosporelings. For peak identification see Table X.

concentration of glutamine estimates based on the area of the glutamine peak have been reduced by 30%. Such estimates leave no doubt that glutamine is predominant among the amino compounds in uredospores (Fig. 27A).

Similar considerations apply to the analysis of the leachate (Fig. 27B), in which glutamine was also predominant. A further complication in the analysis of the leachate arose because of the presence of a very large unidentified peak (X) in the buffer (Fig. 27B) which usually overlapped aspartate (#1) and glutamate (#2). This prevented any meaningful estimates of the levels of aspartate and glutamate in the leachate.

In all chromatograms ammonia and glutathione eluted off the column simultaneously. It was therefore impossible to obtain independent estimates of the levels of NH_3 and glutathione. The NH_3/glutathione peak (#9/20) was usually larger than all other amino compounds except glutamine (Fig. 27). Ten amino acids (alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine, and lysine) were identified and quantified with confidence.

Where possible (see above) the amount of each amino acid was expressed as nM per mg spore dry weight. There was some unavoidable loss of heat shocked uredosporelings during transfer from the germination medium to the extraction vessel because the shorter, differentiated germtubes did not interlock to form a mat. The loss was visually estimated to

be not greater than 15%. Calculations of the amino acid levels per mg dry weight of uredospores were based on the dry weight of the original sample (2.78 mg) and are therefore too low by a maximum of 15%.

Two unknown peaks eluted from the column shortly after the amino acid phenylalanine (#16). Based on results reported by Dwyer et al. (1987) these peaks may be identified tentatively as tryptophan (Trp) and ornithine (Orn). These compounds appeared soon after germination occurred; they were detected in the germinating uredospores and in the leachate from 20-h-old non-shocked uredosporelings.

4.6.3 Exogenous Free Amino Acids: Resting Spore Wash

The buffer in which resting uredospores were washed contained the early-eluting amino acids. These appeared to be associated with the spore wall. Although there was some overlap of aspartic and glutamic acids with the unidentified peak (X) found in the buffer, it was possible to estimate the amounts of these amino acids by approximating the area of the peak. An estimate of glutamine was obtained as described earlier (page 77). The results are shown in Table XI. The amino acids washed off the spores were, in order of predominance, glutamine, alanine, glutamate, aspartate, and tyrosine. An unknown compound with a retention time of 6.66 minutes was also present in the spore wash. Since it was unknown it could not be quantified by its comparison with a

Table XI. Free amino acid composition of the buffer wash from ungerminated uredospores of P. graminis tritici.

Amino Acid	nM/mg spore dry weight[a]	Percent
Ala	0.57 ±.07	26.0
Pro	+	-
Tyr	0.25 ±.06	11.4
Val	+	-
Met	+	-
Cys	ND	-
Ile	+	-
Leu	+	-
Phe	+	-
Lys	ND	-
Glm	0.72 ±.04	32.9
HomoSer	ND	-
Arg/His/Thr[a]	++	-
Unknown*	+	-
Asp	0.27 ±.03	12.3
Glu	0.38 ±.06	17.4
Total	2.19 ±.26	100.0

[a] Each value represents the mean and range for two separate experiments. + = trace amounts, ++ = greater than trace amounts (0.04 nM/mg < tr > 0.12 nM/mg). ND - not detected. [a]see text. *calculated assuming a RF of 4256 (average value for 14 different amino acids).

standard. Therefore it was assigned an RF of 4256 which is an average value calculated from the RF of 14 different amino acids. In addition, small peaks corresponding to the retention times of one or more of arginine, histidine, and threonine were detected (Table XI). It was impossible to tell which of these were actually present. Cysteine, homoserine, and lysine were not detected.

4.6.4 Endogenous Free Amino Acids: Extracts of Unwashed Resting Uredospores

In order of prevalence the amino compounds in unwashed resting uredospores were alanine, the unknown, glutamic acid, and glutamine followed by smaller amounts of several other amino acids (Table XII). Alanine accounted for 30% and the unknown accounted for 22% of the total free amino acids present. The ratio of glutamic to aspartic acid was 12 (2.4/0.2). Cysteine and lysine were not detected.

Comparison of the appropriate columns in Tables XI and XII shows that the total amino acids washed off resting uredospores (Table XI) account for only 16.5% (2.19 x 100 /13.3) of the total amino acids extracted from unwashed resting spores.

Table XII. The endogenous free amino acid level in unwashed resting uredospores (S), and in non-shocked uredosporelings of P. graminis tritici after 8 h (N8S), and 20 h (N20S)

Amino Acid	nM/mg spore dry weight[a]			Ratio	
	S	N8S	N20S	N8S/S	N20S/S
Ala	4.04 ±.47	4.99 ±.71	2.50 ±.30	1.2	0.6
Pro	0.20 ±.0	2.20 ±.29	1.10 ±.13	11.0	5.5
Tyr	+	2.47 ±.04	2.29 ±.64	27.4	25.4
Val	0.43 ±.0	1.80 ±.43	1.23 ±.17	2.9	2.7
Met	+	2.54 ±.07	2.09 ±.42	50.8	41.8
Cys	ND	7.09 ±.39	3.19 ±.77	(>1000)	(>600)
Ile	0.25 ±.01	2.16 ±.15	1.07 ±.17	8.6	4.3
Leu	0.23 ±.02	1.93 ±.33	1.50 ±.54	8.4	6.5
Phe	0.29 ±.02	2.93 ±.31	3.10 ±.58	10.1	10.7
Lys	ND	1.17 ±.60	1.05 ±.02	(>200)	(>200)
Glm	2.23 ±.30	6.99 ±.84	10.74 ±1.2	3.1	4.8
HomoSer	++	++	++	-	-
Arg/His[a]	++	++	++	-	-
Unknown*	2.98 ±.75	2.47 ±.10	2.95 ±.79	1.0	1.2
Orn	ND	++	++	-	-
Trp	ND	++	++	-	-
Asp	0.2 ±.0	2.2 ±.7	1.5 ±.2	22.0	15.0
Glu	2.4 ±.1	10.0 ±2.9	4.1 ±.8	4.2	1.7
Total	13.3 ±1.7	50.9 ±7.6	38.4 ±6.7	3.8	2.9
Glu:Asp	10:1	5:1	3:1	-	-

[a] Each value represents the mean and range for two separate experiments. + = trace amount, ++ = greater than a trace (0.04 nM/mg < tr > 0.12 nM/mg). ND - not detected. [a]see text. *calculated assuming a RF of 4256 (average value for 14 different amino acids.

4.6.5 Effect of Heat Shock on the Free Amino Acids in Germinated Uredospores and in the Leachate After Germination

Unwashed uredospores were placed on Ca-K buffer at 0 h at 19^0 C, heat shocked at 29^0 C from 2 h to 3.5 h and harvested at either 8 h or 20 h. Non-shocked controls were subjected to the same protocol but were maintained at 19^0 C throughout the germination period. Uredosporelings and leachates were analyzed separately. The results are presented in Tables XII, XIII, and XIV.

The data in Table XII show that the endogenous free amino acid level in non-shocked uredospores at 8 h was 3.8-fold, and at 20 h was 2.9-fold that in unwashed resting uredospores. Among the individual amino acids, alanine and the unknown compound showed relatively little change on germination but all the other amino compounds increased markedly. Large relative increases occurred in cysteine and lysine, which were below the limit of detection in resting uredospores. The ratio of glutamate to aspartate decreased sharply on germination.

Table XIII shows that, while the endogenous free amino acid level in heat shocked uredospores increased approximately 2-fold at 8 h, there was no further increase at 20 h. Among the individual amino compounds there were decreases (about 50%) in alanine and the unknown compound. Valine was relatively unchanged. All other amino compounds increased significantly, particularly cysteine and lysine. In general

Table XIII. Endogenous free amino acid content of unwashed resting uredospores (S), and heat shocked uredosporelings of P. graminis tritici after 8 h (H8S), and 20 h (H20S).

Amino Acid	nM/mg spore dry weight[a]			Ratio	
	S	H8S	H20S	H8S/S	H20S/S
Ala	4.04 ±.47	1.80 ±.03	1.40 ±.01	0.5	0.4
Pro	0.20 ±.0	0.66 ±.01	0.46 ±.0	3.3	2.3
Tyr	+	0.93 ±.03	1.20 ±.16	10.3	13.3
Val	0.43 ±.0	0.53 ±.13	0.66 ±.03	1.2	1.5
Met	+	0.56 ±.11	0.84 ±.02	11.2	16.8
Cys	ND	2.89 ±.12	1.45 ±.03	(>600)	(>300)
Ile	0.25 ±.01	0.80 ±.05	0.69 ±.01	3.2	2.8
Leu	0.23 ±.02	1.93 ±.19	1.49 ±.02	8.4	6.5
Phe	0.29 ±.02	1.41 ±.16	1.44 ±.11	4.9	5.0
Lys	ND	0.25 ±.11	0.44 ±.11	(>60)	(>80)
Glm	2.23 ±.30	8.69 ±.89	8.38 ±.35	3.9	3.8
HomoSer	++	++	++	-	-
Arg/His[a]	++	++	++	-	-
Unknown*	2.98 ±.75	1.43 ±.18	1.54 ±.48	0.5	0.5
Orn	ND	++	++	-	-
Trp	ND	+	++	-	-
Asp	0.2 ±.0	1.7 ±.4	1.0 ±.1	17.0	10.0
Glu	2.4 ±.1	4.5 ±1.0	3.5 ±.0	1.9	1.5
Total	13.3 ±1.7	28.1 ±.16	24.5 ±.76	2.1	1.8
Adjusted Total	11.1[b]	32.3[c]	28.3[c]	-	-
Glu:Asp	10:1	3:1	4:1	-	-

[a]Each value represents the mean and range for two separate experiments. + = trace, ++ = greater than a trace (0.04 nM/mg < tr > 0.12 nM/mg). ND - not detected. [a]see text.
*calculated assuming a RF of 4256 (average value for 14 different amino acids. [b]measured endogenous amino acids minus the amino acids in the resting spore wash. [c]measured amounts plus 15% to compensate for uredospore losses during transfer.

Table XIV. The free amino acid composition of leachate emanating from heat shocked (H) and non-shocked (N) uredosporelings of P. graminis tritici after an 8 h (H8L and N8L), and 20 h (H20L and N20L) incubation period.

Amino Acid	nM/mg spore dry weight[a]			
	N8L	H8L	N20L	H20L
Ala	0.60 ±.21	0.70 ±.01	4.52 ±.07	0.91 ±.11
Pro	1.78 ±.17	2.99 ±.59	2.81 ±.43	2.64 ±.24
Tyr	0.29 ±.14	+	1.21 ±.13	0.33 ±.08
Val	0.60 ±.01	0.85 ±.71	1.91 ±.28	+
Met	+	+	1.36 ±.03	+
Cys	ND	ND	5.42 ±.13	+
Ile	+	+	1.61 ±.03	+
Leu	+	+	1.17 ±.03	+
Phe	+	ND	2.06 ±.05	ND
Lys	+	+	0.66 ±.01	ND
Glm	2.40 ±.20	2.39 ±.33	9.93 ±.00	9.91 ±1.17
HomoSer	++	++	++	++
Arg/His[a]	++	++	++	++
Unknown*	ND	ND	3.31 ±.35	ND
Trp	ND	ND	++	ND
Orn	ND	ND	++	ND
Total: Leachate (L)	5.67 ±.30	6.93 ±.98	35.97 ±.02	13.79 ±.95
Wash (W)[g]	2.19 ±.26	2.19 ±.26	2.19 ±.26	2.19 ±.26
L - W	3.48	4.74	33.78	11.60

[a]Each value represents the mean and range for two separate experiments. + = trace, ++ greater than a trace, (0.04 nM/mg < tr > 0.12 nM/mg). ND - not detected. [a]see text. *calculated assuming a RF of 4256 (average value for 14 different amino acids. [g]from Table XI.

the increases in individual amino compounds were smaller than in non-shocked spores. Moreover the amino acid level in heat shocked spores was only 55% (28.1 x 100 /50.9) of that in non-shocked spores at 8 h and 64% (24.5 x 100 /38.4) at 20 h. It is thus clear that heat shock decreases protein hydrolysis in germinating uredospores.

Paraphrasing the well known song, we may ask "Where have all the amino acids gone?". The data in Table XIV, which presents the analyses of the leachates, provides the answer. By 8 h non-shocked and shocked uredospores had lost approximately the same total amount of amino compounds to the germination medium, ca 5.7 and 7.0 nM/mg dry weight respectively. In contrast, by 20 h the non-shocked uredospores had lost nearly 36.0 and the shocked uredospores only ca 13.8 nM/mg spore dry weight respectively. Both non-shocked and heat shocked uredosporelings lost about equal amounts of glutamine. At 20 h losses in alanine, cysteine, phenylalanine, and the unknown were much greater from the non-shocked sporelings.

It is important to note that the leachate from unwashed germinating uredospores presumably includes the amino compounds which could be washed off the resting uredospores (Table XI). Differences in the total amounts of amino compounds found in the leachates and in the spore wash are therefore also shown in Table XIV.

5. DISCUSSION

5.1 Temperature Requirement for the Differentiation of P. graminis tritici Uredosporelings

Maheshwari et al. (1967a) demonstrated that greater than 90% of P. graminis tritici uredosporelings undergo complete differentiation on Ca-K buffer in response to a 1.5 h heat shock of 30^0 C administered 2 h after seeding. Wisdom (1977) obtained only 5% differentiation using the methods described above. The effect of the heat shock temperature was not investigated.

The results presented in this thesis show that : (1) Differentiation has a sharp temperature optimum. Aberrations one degree above or below the optimum heat shock temperature significantly reduced (by greater than 40%) the amount of total sporelings forming a complete set of infection structures. (2) The optimum heat shock temperature varies slightly for different spore lots. When the temperature of the heat shock was optimised up to 70% differentiation was obtained. These results indicate that Wisdom's failure to obtain better than 5% differentiation on Ca-K buffer was most likely due to the use of a sub-optimal heat shock temperature.

5.2 The Influence of Nutrients on Uredospore Differentiation

Since Wisdom (1977) obtained only 5% differentiation on Ca-K buffer she abandoned the buffer in favour of her nutrient-enriched MPG medium. Using MPG in conjunction with a 1.5 h heat shock of 30^0C, Wisdom obtained up to 80% differentiation.

Williams (1971) had earlier shown that nutrients stimulate differentiation and that the nutrient stimulus is more effective when a heat shock is applied. The results obtained in the present study support this claim. Moreover, this response of the uredosporelings to nutrients in the germination medium was found to be non-additive. It was also found that n-nonyl alcohol not only stimulates germination but also increases the proportion of germtubes forming infection structures.

Results from preliminary experiments indicated that MPG was superior to Ca-K buffer as a differentiation medium for *P. graminis tritici* uredosporelings. The precise temperature optimum for the heat shock observed with sporelings germinated on Ca-K buffer (page 87) was not observed with sporelings germinated on MPG. On MPG, a single given temperature (30^0 C) consistently induced the differentiation of a high percentage of uredosporelings.

Ca-K buffer appeared to be the most effective component of MPG. Baker *et al*. (1987) reported that Ca^{+2}, but not K^+,

stimulated the germination of Uromyces phaseoli uredospores. It is also possible that calcium plays a key role in uredosporeling differentiation. One of the many functions of calcium in biological systems is the regulation of cell membrane permeability to water and ions; low calcium generally increases permeability whereas high calcium decreases it. The potassium phosphates supply the hyphal cell with phosphorus. Phosphorus is required for the formation of nucleic acids and phospholipids, as well as a key molecule of energy metabolism, ATP.

Peptone in distilled water was a poor medium for differentiation. In combination with glucose and Ca-K buffer peptone significantly increased the number of sporelings that formed infection structures (up to 77%). Bacto-peptone contains a variety of simple nitrogenous compounds (including reduced organic sulphur), ions, minerals, and a negligible amount of protease and complex nitrogenous compounds (Difco Laboratories 1953).

Glucose is a potential source of energy for hyphal growth. In the presence of glucose (dissolved in glass-distilled water) the germtube did not appear to respond to heat shock but continued linear growth without the formation of infection structures. Although glucose alone was ineffective as a differentiation medium it played an important role as a constituent of MPG. According to Manners and co-workers (1982) glucose is readily absorbed and metabolized by

the rust hyphae. The carbon appeared in endogenous pools of free glucose, amino acids and phosphate esters of trehalose and glucose (Reisener et al. 1961).

Previous studies on the enzymatic constitution of uredospores of the wheat stem rust have indicated the presence of complete enzyme systems for the metabolism of amino acids, lipids, and carbohydrates (see Shaw 1964). The presence of nutrients in the medium may provide the uredosporeling with metabolites that assist with the processes involved in germtube morphogenesis.

5.3 The Timing of Essential Protein Synthesis

The timing of RNA and protein synthesis during differentiation has been described by Dunkle et al. (1969) and Wisdom (1977). It has been shown that new polypeptides appear during the differentiation of non-shocked bean rust uredosporelings (Huang and Staples 1982), and the differentiation of heat shocked wheat stem rust uredosporelings (Kim et al. 1982a). Although not differentiated, heat shocked flax rust uredosporelings synthesized a number of heat shock proteins (Shaw et al. 1985). These results have been discussed in greater detail in the literature review (pages 15).

The effect of puromycin on differentiation was reinvestigated under the optimum heat shock conditions

described earlier (page 87). Puromycin is recognized as an effective inhibitor of protein synthesis. It is a structural analogue of the aminoacyl adenosine, the amino-acid-bearing end of transfer-RNA. It binds readily to the aminoacyl site of the large ribosomal subunit and forms a complex with the elongating peptide chain. The peptidyl-puromycin complex prevents further amino acid incorporation. The complex dissociates from the ribosome and chain elongation is terminated (Gale et al. 1972).

During the present study puromycin was added to, or removed from, the germination medium (Ca-K buffer) at various stages during sporeling development. The effect of puromycin on germtube morphogenesis was dependent on the timing and duration of the puromycin treatment. The addition of puromycin to Ca-K buffer at 3.5 h followed by its removal at 8 h caused a significant reduction in the proportion of infection pegs forming vesicles. Although the vesicles were visible after 10 h their formation appeared to be preceded by the synthesis of essential proteins. The results suggest that these proteins are synthesized 2 h to 6.5 h prior to vesicle formation.

When puromycin was present in the germination medium for the entire incubation period (0 h to 16 h) germtube differentiation was visibly affected: (1) The complete differentiation of uredosporelings was prevented. (2) The proportion of germtubes forming appressoria was significantly

reduced. (3) The appressoria were predominantly irregular in form. (4) The formation of a septa between the appressoria and the germtube usually failed to occur. (5) Nuclear division was rarely observed, but in the rare event of division the pairing of daughter-nuclei did not occur. The capacity of the inhibited germlings to form infection structures appeared to recover partially when puromycin was removed from the germination medium.

These results suggest the existence of differentiation-specific proteins and support the claim that these proteins are stage-specific (Huang and Staples 1982). It may be concluded that essential proteins are synthesized throughout the entire process of differentiation, including the formation of the appressoria.

The presence of puromycin in the germination medium throughout the incubation period usually prevented the division of germtube nuclei. Nuclear division is generally preceded by the replication of nuclear DNA. Prior to the onset of the replication of DNA certain enzymes such as, thymidine kinase, thymidylate synthetase and others, must increase within in the cell. The absence of nuclear division observed in this study suggests that these enzymes are synthesized prior to the formation of the appressorium. In rare cases the enzymes appear to be present in sufficient amounts to enable replication and division to occur.

5.4 Nuclear Behaviour Accompanying Germination and Differentiation

The two nuclei of nondifferentiated uredosporelings did not divide but appeared to remain in perpetual interphase throughout the entire observation period of 20 h. In differentiating uredosporelings nuclear division was a regular event. Its occurrence was closely associated with the timing of infection structure development. The cytological events taking place during the first 24 h of germination and differentiation are summarized in Fig. 28.

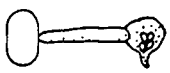
A characteristic pattern of nuclear behaviour was observed in differentiating uredosporelings. The protoplasm migrated into the developing infection structures leaving an empty space in the proximal regions of the germtube. Paired daughter nuclei migrated in tandem with the cytoplasm. The 4 to 6 nuclei observed in the appressorium migrated into the vesicle as a closely associated group. This was not observed by Wisdom (1977) who reported that the movement of the 4 nuclei from the appressorium into the developing vesicle was not synchronous. She noted that one pair moved into the vesicle while the other pair remained in the appressorium for up to 10 h. Moreover, Wisdom (1977) did not report the potential for a second nuclear division in the appressorium. In the present study 6 nuclei were commonly observed moving into the developing infection peg. Migration of nuclei into

2 h 

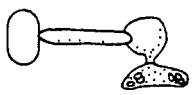
Two expanded nuclei migrate down the germtube toward its apex.

5 h 

The first round of mitosis is generally completed within the mature appressorium.

7 h 

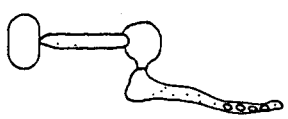
The cytoplasm moves into the infection peg with the nuclei. A second division occurs either prior to or during nuclear migration.

10 h 

The mature substomatal vesicle contains six unexpanded nuclei. A third mitotic division may occur to yield eight nuclei.

13 h 

Eight, six, or four nuclei enter the infection hypha, occasionally a nuclear pair remains in the vesicle.

24 h 

Four or fewer nuclei migrate along the infection hypha.

Fig. 28. Diagrammatic representation of the cytological events taking place during the differentiation of uredosporelings of P. graminis tritici, race C17. The times given are approximate.

the developing infection structures was characterized by a slight change in nuclear shape. The nuclei appeared to be "tear drop" in shape as if they were "pulled" into the infection structures. When a break occurred in the germtube the nuclei were extruded along with the cytoplasm. In some cases, when nuclei were migrating into the infection hypha, the foremost nucleus appeared brighter than the following ones. Wisdom (1977) described an analogous situation in which a single intensely Feulgen-positive nucleus preceded the other nuclei into the infection hypha. In the rare event of a bipolar vesicle forming, two "bright" nuclei could be seen. One nucleus was situated on either side of the vesicle and adjacent to the initials of the infection hyphae. Finally 2 or more nuclei appeared to either break down or coalesce within the infection hypha, so that after 24 h the hypha contained up to a maximum of four distinct nuclei (Fig. 28).

5.4.1 Staining with DAPI

DAPI (4', 6-diamidino-2-phenylindole) is a DNA-specific fluorochrome. It binds relatively specifically to AT residues of double-stranded DNA and exhibits a much enhanced fluorescence in the association (Otto and Tsou 1985). This fluorescence is not observed with RNA or protein (Coleman et al 1981). Chemically, it is suggested that the AT-specificity resides with both the guanidine group and the benzimidazole or indole ring, which may bind to the purine of

adenosine through base stacking (Otto and Tsou 1985).

DAPI binds to DNA and fluoresces in proportion to the amount of DNA present. The relative DNA content per nucleus can be read *in situ* by measuring the intensity of fluorescence with a microspectrofluorometer (Coleman *et al* 1981). The enhanced intensity of fluorescence observed in the leading nucleus (preceded the other nuclei into the infection hypha) may suggest that this nucleus is not haploid but contains additional DNA.

DAPI reacted rapidly with the sample and within minutes one could easily assess nuclear state and identify nuclear abnormalities. The technique appeared to be useful for determining the numbers and positions of nuclei and their division sites within the infection structures. The stain is water-soluble and has been used successfully as a vital dye in studies on red algae and pollen development (Goff and Coleman 1984, Coleman and Goff 1985). It should have further application in assessing the effects of a number of parameters (e.g. nutrition, fungicides, and host genotype manipulation) on the nuclear behaviour of the rust pathogen.

5.5 The Effect of Heat Shock on the Amounts and Kinds of Free Amino Acids in Germinated Uredospores and Their Leachates

The results presented in Tables XI to XIV show clearly that heat shock decreases the size of the endogenous pool of free amino acids and the extent to which uredosporelings lose amino acids to the medium. The results are summarized in Table XV which presents a "balance sheet" showing the size of the endogenous and leachate amino acid pools.

Under the conditions employed only endogenous reserves are available to the germinating uredospores. The amino acid data (Table XV) therefore clearly show that there is a net hydrolysis of protein during germination, leading to an increase in the level of endogenous free amino acids. These results are consistent with data obtained from pulse chase experiments (Kim et al. 1982a) which demonstrated that the majority of uredospore proteins are turned over during germination. The level of total amino acids remained relatively unchanged in the heat shocked sporelings from 8 h to 20 h (39.3 to 42.0 nM/mg), whereas the level of total amino acids in non-shocked sporelings increased from 55.8 nM/mg at 8 h to 74.4 nM/mg at 20 h. The total free amino acid level (endogenous and leachate) associated with non-shocked uredosporelings was thus 1.8-fold (74.4/42.0) higher than the amount of amino acids associated with heat shocked uredosporelings at 20 h. It is thus clear that heat shock

Table XV. The distribution of the free amino compounds associated with resting spores, non-shocked 8- (N8S) and 20-h-old (N20S) uredosporelings, and heat shocked 8- (H8S) and 20-h-old (H20S) uredosporelings. The results are expressed as nM amino acid per mg spore dry weight.

	S	N8S	H8S	N20S	H20S
Endogenous	11.1[b]	50.1	32.3[c]	38.4	28.2[c]
Leachate[d]	-	5.67	6.95	36.0	13.8
Wash	2.19	-	-	-	-
Total	13.3	55.8	39.3	74.4	42.0

[b]measured endogenous amino acids minus the amino acids in the resting spore wash. [c]measured amounts plus 15%, see Table XII. [d]includes amino acids in resting spore wash (2.19 nM/mg).

decreases the amount of protein hydrolysis in germinating uredospores.

The results show further that there is no net protein synthesis during the formation of infection structures induced by heat shock. Nevertheless, protein synthesis is increased relative to protein hydrolysis by comparison with the relative rates of these two processes in nondifferentiating (non-shocked) uredosporelings. These results and the conclusions drawn from them are consistent with the effect of puromycin on differentiation. The inhibition of protein synthesis by puromycin inhibits differentiation but does not appear to decrease the linear growth of nondifferentiated (non-shocked) uredosporelings. The results are also consistent with the observed effects of heat shock on the incorporation of [^{35}S]-methionine into newly synthesized heat shock proteins in germinating flax rust uredosporelings reported by Shaw et al. (1985).

A high proportion of the total amino acid pool was lost to the medium during germination, particularly in non-shocked uredosporelings. The extent of this leakage indicates that the lost amino acids must originate from the hydrolysis of protein. Daly and his colleagues (1967) also found that in non-shocked uredospores of P. graminis tritici a considerable portion of the free amino acids arising from protein degradation are lost to the medium and that only a small portion are utilized for the resynthesis of proteins. The

results in this thesis show that the loss of free amino acids from non-shocked sporelings to the medium is 2.6 (36.0/13.8) times greater than the loss from heat shocked sporelings. As spore germination progressed (from 8 h to 20 h) the loss of amino acids from non-shocked uredosporelings to the medium increased and appeared less selective than the loss from heat shocked sporelings.

In contrast, heat shock markedly decreased the loss at 20 h of most of the amino acids listed on Table XIV. Particularly striking decreases occurred in the losses of alanine, cysteine, phenylalanine and an unknown amino acid. On the other hand heat shock had no significant effect on the losses of proline and glutamine. The loss of proline and glutamine is not surprising since they are readily formed via the conversion of many amino acids and metabolites. Proline commonly functions as a long-distance transport compound for carbon. Proline is an ideal translocation molecule by virtue of its high metabolic capabilities (eg. to form glutamate, ã-ketoglutarate, and pyruvate via succinate) (Miflin 1977). Glutamine, on the other hand is the principle nitrogen donor for many biosynthetic reactions, and its formation is a detoxification mechanism for the removal of ammonia.

Individual amino acids were lost to the medium at different rates and in different amounts (Table XV). For example, non-shocked sporelings lost 0.6 nM/mg alanine in 8 h and 4.5 nM/mg in 20 h (ie. the loss at 20 h was 7.5 times the

loss at 8 h). Non-shocked sporelings also lost 2.4 nM/mg glutamine at 8 h and 9.9 nM/mg at 20 h (ie. the loss at 20 h was 4.2 times the loss at 8 h). On the other hand the loss of alanine from heat shocked sporelings at 20 h was 1.14 times the loss at 8 h and the loss of glutamine at 20 h was 4.15 times the loss at 8 h. Comparing alanine and glutamine we see that they were each lost at different rates in non-shocked and heat shocked sporelings. Comparing non-shocked and heat shocked sporelings we see that alanine was lost at different rates but glutamine at the same rate in the two sets of sporelings. Similar comparisons can be made for the other amino acids from the data in Table XV. It follows that if amino acids are lost from uredosporelings via a membrane defect, the defect does not affect the loss of all amino acids to the same extent. Therefore, the loss of amino acids from the uredosporeling to the medium is considered to be selective. In summary, the composition of each amino acid pool (exogenous and leachate pools) is unique. The percent composition of each amino acid to the total amino acids in each pool is: (1) different, (2) alters as germination progresses through 8 h to 20 h, and (3) is altered by heat shock.

The question which next arises is the mechanism of the effect of the heat shock in selectively decreasing the loss of amino acids. While heat shock promotes the synthesis of certain highly conserved heat shock proteins and depresses the

synthesis of other proteins (Shaw et al. 1985), the exact mechanisms involved are unknown. With respect to the leakage of amino acids into the medium it is possible that heat shock directly affects on the integrity and hence the permeability of the cell membranes. Alternatively it may alter amino-acid transport processes across the membranes. The data in this thesis do not provide any direct evidence on which to base an answer to these questions.

Irrespective of the effect of heat shock, the growth of the germtube is accompanied by the loss of amino acids to the medium. It is possible that exogenous amino acids arise from either the mechanical rupture or enzymatic degradation of the germtube cell wall. Although germtube lysis was not observed it is likely that some degradation of the hyphal wall occurs during germination. The break down of the germtube wall would facilitate the loss of amino acids as well as other cell metabolites and enzymes. In addition, it is possible that the loss of amino acids to the medium is the result of a structural or chemical defect in the cell membrane. A membrane defect may lead to the loss of selective permeability and/or an alteration in amino acid transport systems.

Scott and Maclean (1969) have suggested that the rust fungi resemble mammalian cell cultures in that both cell lines appear to have a membrane defect allowing the loss of newly synthesized metabolites into the medium. The appearance of free amino acids within the leachate implies the absence of

regulation of permeability in the hyphal membrane (Scott and Maclean 1969). If this assumption is valid it may explain the high uredospore density usually required for initiating normal saprophytic growth. It is well known that radioactive amino acids fed to non-shocked germinating uredospores are taken up and incorporated into protein (Shaw 1964, Shaw et al. 1985). At high cell densities the leaked nutrient can reenter another cell in close proximity, thus maintaining an effective intercellular level by cross feeding between cells. At low cell densities a metabolite may be lost into the medium at a rate equal to its rate of synthesis. A critical population density is able to build up an extracellular concentration of the metabolite that is in equilibrium with the minimum intracellular level, before the cells die of a specific nutritional deficiency (Eagle and Piez 1962).

Amino acids have important physiological roles which govern many metabolic processes (e.g. growth and enzyme activity). A number of interesting avenues of research have come to this authors attention during this study: the effect of exogenous amino acids on germination and differentiation; the effect of some amino acids (pathway products) on extractable enzyme activities; the effect of exogenous amino acids on enzyme activity (derepression/repression of genes); and to explore the phenomenon of growth inhibition due to amino acid imbalances. Although research is being pursued in these areas most of the work has focussed on organisms other

than the rust fungi.

Most of the recent work published is concerned with the timing and the products of protein synthesis; however, the activation of protein synthesis during germination and the formation of infection structures has not been well documented. Given the information contained in the present study it would be of interest to determine the amount of charged tRNA for each amino acid. To significantly affect protein synthesis the absence of a free amino acid would result in the absence of the aminoacyl-tRNA.

Although some growth of several strains of wheat stem rust has been obtained axenically, only the Australian race, 126-ANZ-67, cultured by William's group (1966, 1967) has been found to grow vigorously. Analyses of the amino acid composition of the leachate may provide a useful basis for formulating the amino acid component of media for the axenic culture of stem rust. Moreover, it would also be of interest to extract and purify the free amino acid fraction from germinating uredospores. This fraction could be used to provide the amino acid component of an axenic medium for rust culture.

6. SUMMARY

(1) The percent germination of P. graminis tritici, race C17, uredospores and the proportion of germtubes forming complete infection structures was augmented by n-nonyl alcohol.

(2) A precisely timed heat shock and exogenous nutrients stimulate differentiation. This stimulus is most effective in the presence of n-nonyl alcohol.

(3) The heat shock temperature required to induce maximum differentiation has a very precise optimum. Variations one degree above or below this optimum for a given spore lot reduced the percent differentiation by greater than 40%.

(4) Although the optimum temperature of the heat shock varied slightly depending on the particular spore lot the sensitivity of sporeling development to temperature changes is remarkably constant.

(5) Compared to Ca-K buffer, MPG was a superior germination and differentiation medium.

(6) Ca-K buffer was the most effective component of MPG.

(7) Uredosporeling differentiation occured as a series of precisely timed morphological, cytological, and physiological events.

(8) DAPI staining is a simple, rapid, and reproducible way to assess nuclear behaviour in P. graminis tritici uredosporelings.

(9) Nuclear division was a regular event in differentiating uredospores. Its occurrence was closely associated with the timing of infection structure development.

(10) Nuclear division was a rare event in germinating (non-shocked) uredospores.

(11) Essential, presumably differentiation-specific proteins, were synthesized from the onset of germination to the completion of differentiation. These proteins are required for the formation of appressoria, vesicles and, infection hyphae.

(12) Spore germination was accompanied by a rapid decrease in the glutamic:aspartic acid ratio (from 20:1 to 3:1).

(13) There is a net hydrolysis of protein during germination, leading to an increase in size of the endogenous pool of free amino acids and to an increased leakage of amino acids to the germination medium.

(14) Relative to non-shocked uredosporelings, heat shock decreased both the size of the endogenous pool of amino acids and the extent to which uredosporelings lose amino acids to the medium.

(15) There was no net protein synthesis during the formation of infection structures induced by heat shock.

(16) A high proportion of the total amino acid pool was lost to the medium during germination, particularly in non-shocked uredosporelings.

(17) Free cysteine was detected in the leachate isolated from non-shocked sporelings only.

(18) The loss of amino acids to the germination medium is selective, particularly in heat shocked uredosporelings.

(19) The composition of the amino acid pool in the leachate may be a useful guide in formulating media for the axenic culture of P. graminis tritici.

7. LITERATURE CITED

Allen, R.F. 1923. A cytological study of infection of Baart and Kanred wheats by Puccinia graminis tritici. J. Agric. Res. 23: 131-152.

Allen, P.J. 1972. Specificity of the cis-isomers of inhibitors of uredospore germination in the rust fungi. Proc. Natl. Acad. Sci. USA. 69: 3497-3500.

___. 1976. Control of spore germination and infection structure formation in the rust fungi. In Physiological Plant Pathology, eds. R. Heitefuss, P.H. Williams, 51-78. New York: Springer-Verlag.

Artemenko, E.N., Umnov, A.M., and Chkanikov, D.I. 1980. Changes in the level of indolyl-3-acetic acid and possible paths of its regulation in leaves of wheat infected with stem rust. Sov. Plant Physiol. (Eng. Transl.) 27: 447-451.

Ashburner, M. 1982. The effects of heat shock and other stress on gene activity: an introduction. In Heat Shock: from Bacteria to Man, eds. M.J. Schlesinger, M. Ashburner, A. Tissiers, 1-9. New York: Cold Spring Harbour Lab. Press.

Baker, C.J., Tomerlin, J.R., Mock, N., Davidson, L., and Melhuish, J. 1987. Effects of cations on germination of urediniospores of Uromyces phaseoli. Phytopathology. 77: 1556-1560.

Bell, A.A., and Daly, J.M. 1962. Assay and partial purification of self-inhibitors of germination from uredospores of the bean rust fungus. Phytopathology 52: 261-266.

Bhattacharya, P.K., Naylor, J.M., and Shaw, M. 1965. Nucleic acid and protein changes in the wheat leaf nuclei during rust infection. Science 150: 1605-1607.

Bidwell, R.G.S. 1979. Plant Physiology, pp 192-227. New York: Macmillian Pub. Co.

Boedijn, K.B. 1965. Trypan Blue as a stain for fungi. Stain Tech. 31: 115-116.

Bushnell, W.R. 1976. Growth of races 38 and 17, P. graminis tritici on artificial media. Can. J. Bot. 54: 1490-1498.

Bushnell, W.R. 1984. Structural and physiological alterations in susceptible host tissue. In The Cereal Rusts .I. Origins, Specificity, Structure, and Physiology, 477-507. New York: Academic Press Inc. Ltd.

Caltrider, P.G., and Gottlieb, D. 1963. Respiratory activity and enzymes for glucose catabolism in fungus spores. Phytopathology 53: 1021-1030.

Chakravorti, B.P. 1966. Attempts to alter infection processes and aggressiveness of Puccinia graminis tritici. Phytopathology 56: 223-229.

Chakravorti, A.K., Shaw, M., and Scrubb, L.A. 1974. Changes in ribonuclease activity during rust infection. I. Characterization of multiple molecular forms of ribonuclease from flax rust grown in host-free media. Physiol. Plant Path. 4: 313-334.

Cochrane, J.C., Rado, T.A., and Cochrane, V.W. 1971. Synthesis of macromolecules and polyribosome formation in early stages of spore germination in Fusarium solani. J. Gen. Microbiol. 65: 45-55.

Coffey, M.D., and Shaw, M. 1972. Nutritional studies with axenic cultures of the flax rust Melampsora lini. Physiol. Plant Path. 2: 37-46.

Coffey, M.D., and Allen, P.J. 1973. Nutrition of Melampsora lini and Puccinia helianthi. Trans. Br. Mycol. Soc. 60: 245-260.

Coleman, A.W., Maquire, M., and Coleman, J.R. 1981. Mithromycin and DAPI-staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids and virus particles. J. Histochem. Cytochem. 19: 959-968.

Coleman, A.W., and Goff, L.J. 1985. Applications of fluorochromes to pollen biology. I. Mithromycin and 4',6-diamidino-2-phenylindole (DAPI) as vital stains and for the quantification of nuclear DNA. Stain Tech. 60 (3): 145-154.

Craigie, J.H. 1959. Nuclear behaviour in the diploidization of haploid infections of Puccinia helianthi. Can. J. Bot. 37: 843-855.

Daly, J.M., Knoche, H.W., and Wiese, M.V. 1967. Carbohydrate and lipid metabolism during germination of uredospores of P. graminis tritici. Plant Physiol. 42: 1633-1642.

De Jong, E.J., Eskes, A.B., Hoogstraten, J.G.J., and Zadoks, J.C. 1987. Temperature requirements for germination, germtube growth and appressorium of urediospores of Hemileia vastatrix. Neth. J. Plant Path. 93: 61-71.

Dickinson, S. 1949. Studies in the physiology of obligate parasitism. II. The behaviour of the germtubes of certain rusts in contact with various membranes. Ann. Bot. London 13: 219-236.

___. 1971. Studies in the physiology of obligate parasitism. IX. The measurement of thigmotrophic stimulus. Phytopathol. Z. 73: 347-358.

Difco Laboratories. 1953. Difco Manual 9th Ed., 265. Detroit, U.S.A.: Difco Lab. Inc.

Dube, H.C., and Bordia, S. 1982. Effect of amino acids on pectylitic enzymes produced by Helminthosporium sacchari, the incitant of 'eye-spot' disease of sugarcane. Proc. Nat'l. Acad. Sci. India. B. 52(1): 25-28.

Dunkle, L.D., Maheshwari, R., and Allen, P.J. 1969. Infection structures from rust uredospores: Effect of RNA and protein synthesis inhibitors. Science 163: 481-482.

Dunkle, L.D., and Allen, P.J. 1971. Infection structure differentiation by wheat stem rust uredospores in suspension. Phytopatholgy 61: 649-652.

Dwyer, M.E., Merion, M., and Sousa, K.R. 1987. Applications of a physiological amino acid analysis system for the biological researcher. J. Analysis and Purification Sept: 46-49.

Eagle, H. and Piez, Z. 1962. The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. J. Exp. Med. 116: 29.

Epstein, L., Lacetti, L., Staples, R.C., Hoch, H.C., and Hoose, W.A. 1985. Extracellular proteins and differentiation induction by bean rust uredospore germlings. Phytopathology 75(9): 1073-1076.

Flor, H.H. 1971. Current status of the gene-for-gene concept. Ann. Rev. Phytopath. 9: 275-296.

Foudin, A.S., and Wynn, W.K. 1972. Growth of P.graminis tritici on a defined medium. Phytopathology 62: 1032-1040.

French, R.C., and Weintraub, R.L. 1957. Pelargonaldehyde as an endogenous germination stimulator of wheat stem rust spores. Arch. Biochem. Biophys. 72: 235-237.

Gassner, G., and Franke, W. 1938. Untersuchungen uber den stickstoffhaushalt rostinfizierter Getreideblatter. Ein Beitrag zum problem der Teleutospoenbildung. Phytopath. Z. 5: 517-570.

Goff, L.J.A., and Coleman, A.W. 1984. Elucidation of fertilization and development in a red algae by quantitative DNA microspectrofluorometry. Devel. Biol. 102: 173-194.

Grambow, H.J. 1977. The influence of volatile leaf constituents on the in vitro differentiation and growth of P.graminis tritici. Z. Pflanzenphysiol. Bd. 85: 361-372.

___. 1978. The effect of Nordihydroguajaretic acid on the development of the wheat rust fungus. Z. Pflanzenphysiol. 88: 369-372.

Grambow, H.J., and Grambow, G.E. 1978. The involvement of epicuticular and cell wall phenols of the host plant in the in vitro development of P. graminis tritici. Z. Pflanzenphysiol. 90: 1-9.

Grambow, H.J., and Muller, D. 1978. Nuclear condition, types of hyphal development from differentiating and non-differentiating uredosporelings, and effect of 3,3'-bis-indolylmethane on P. graminis tritici in vitro. Can. J. Bot. 56: 736-741.

Grambow, H.J., and Riedel, S. 1977. The effect of morphologically active factors from the host and nonhost plants on the in vitro differentiation of infection structures of P. graminis tritici. Physiol. Plant Path. 11: 213-224.

Hartley, M.J. and Williams, P.G. 1971a. Interactions between strains of Puccinia graminis f.sp. tritici in axenic culture. Trans. Br. Mycol. Soc. 57:129-136.

___. 1971b. Morphological and cultural differences between races of Puccinia graminis f.sp. tritici in axenic culture. Trans. Br. Mycol. Soc. 57: 137-144.

Heath, I.B., and Heath, M.C. 1978. Microtubules and organelle movements in the rust fungus Uromyces phaseoli var. vignae. Cytobiology 16: 393-411.

Hepper, C.M. 1986. Growth of hyphae from Glomus spores in the presence of sulphur-containing compounds. Mycorrhizae: physiology and genetics. SEM, Dijon, 1-5 July 1985. INRA, Paris: 527-530.

Hess, S.L., Allen, P.J., Nelson, D., and Lester, H. 1975. Mode of action of methyl cis-ferulate, the self-inhibitor of stem rust uredospore germination. Physiol. Plant Path. 5: 107-112.

Hoch, H.C., Staples, R.C., and Bourett, T. 1984. Cell differentiation in Uromyces. Involvement of the cytoskeleton (abstr.). J. Cell Biol. 99: 200a.

Hoch, H.C., and Staples, R.C. 1985. The microtubule cytoskeleton in hyphae of Uromyces phaseoli germlings: Its relationship to the region of nucleation and to the F-actin cytoskeleton. Protoplasma 124: 112-122.

Hoch, H.C., Staples, R.C., Whitehead, B., Comeau, J., and Wolf, E.D. 1987. Signalling for growth orientation and cell differentiation by surface topography in Uromyces. Science 235: 1659-1662.

Howes, N.K., and Scott, K.J. 1972. Sulphur nutrition of P.graminis tritici in axenic culture. Can. J. Bot. 50: 1165-1170.

___. 1973. Sulphur metabolism of P. graminis tritici. J. Gen. Microbiol. 76: 345-354.

Howes, N.K., Kim, W.K., and Rohringer, R. 1982. Detergent-soluble polypeptides extracted from uredospores of four physiologic races of Puccinia graminis f.sp. tritici. Physiol. Plant Pathol. 21(3): 361-366.

Huang, B.F., and Staples, R.C. 1982. Synthesis of proteins during differentiation of the bean rust fungus. Exp. Mycol. 6: 7-14.

Jaffe L.F. 1981. The role of ionic currents in establishing developmental pattern. Phil. Trans. R. Soc. Lond. B. 295: 553-566.

Johansen, D.A. 1940. Plant Microtechnique. New York: McGraw-Hill.

Johnson, L.B., Brannaman, B.L., and Zscheile, F.P. 1968. Protein and enzyme changes in wheat leaves following infection with Puccinia recondita. Phytopathology 58(5): 578-583.

Jones, J.P. 1966. Absorption and translation of ^{35}S in oat plants inoculated with labeled crown rust uredospores. Phytopathology 56: 272-275.

Jones, J.P., and Snow, J.P. 1965. Amino acids released during germination of ^{35}S-labeled crown rust spores (abstr.). Phytopathology 55: 499.

Kasting, R., McGinnis, A.J., and Broadfoot, W.C. 1959. Biosynthesis of some amino acids from sucrose by germinating uredospores of wheat stem rust fungi, race 15B. Nature 184: 1943.

Kim, W.K., and Rohringer, R. 1969. Metabolism of aromatic compounds in healthy and rust-infected primary leaves of wheat. III. Studies on the metabolism of tryptophan. Can. J. Bot. 47(9): 1425-1433.

Kim, W.K., Howes, N.K., and Rohringer, R. 1982a. Detergent-soluble polypeptides in the germinated uredospores and differentiated uredosporelings of wheat stem rust. Can. J. Plant Path. 4: 328-333.

Kim, W.K., Rohringer, R., and Chong, J. 1982b. Sugar and amino acid composition of macromolecular constituents released from walls of uredosporelings of P. graminis tritici. Can. J. Plant Path. 4(4): 317-327.

Kiraly, Z., and Farkas, G. 1962. Biochemical character of resistance in wheat to black stem rust. Review of Applied Mycology Abstr. 704.

Kropft D.L., Caldwell J.H., Gow N.A.R., and Harold F.M. 1984. Transcellular ion currents in the water mold Achyla. Amino acid proton symport as a mechanism of current entry. J. Cell Biol. 99: 486-496.

Kuck, K.H., and Reisener, H.J. 1985. In vitro sporulation of race 32 of Puccinia graminis f.sp. tritici Eriks. and Henn. Physiol. Plant Path. 27(3): 259-267.

Kuhl, J.L., Maclean, D.J., Scott, K.J., and Williams, P.G. 1971. The axenic culture of Puccinia species from uredospores: experiments on nutrition and variation. Can. J. Bot. 49: 201-209.

Levin, I.M. 1985. Dependence of the development rates of wheat stem rust on changing substance transport in the detached leaf treated with phytohormones. Mikologiya i Fitopatologiya 19(3): 256-260.

Macko, V., Staples, R.C., Allen, P.J., and Renwick, J.A.A. 1971. Identification of the germination self-inhibitor from wheat stem rust uredospores. Science 173: 835-836.

Macko, V., Staples, R.C., Renwick, J.A.A., and Pirone, J. 1972. Germination self-inhibitors of rust uredospores. Physiol. Plant Pathol. 2: 347-355.

Macko, V., Staples, R.C., Yaniv, Z., and Granadas, R.R. 1976. Self-inhibitors of fungal spore germination. In: The Fungal Spore: Form and Function, eds. D.J. Weber, W.M. Hess, 73-100. New York: John Wiley and Sons.

Macko, V., Renwick, J.A.A., and Rissler, J.F. 1978. Acrolein induces differentiation of infection structures in the wheat stem rust fungus. Science 199: 442-443.

___. 1982. Axenic culture and metabolism of rust fungi. In The Rust Fungus, eds. K.J. Scott and A.K. Chakravorty, 37-81. London: Academic Press.

Maheshwari, R., Allen, P.J., and Hildebrandt, A.C. 1967a. Physical and chemical factors controlling the development of infection structures from uredospore germtubes of rust fungi. Phytopathology 57: 855-862.

___. 1967b. The cytology of infection structure development in uredospore germtubes of Uromyces phaseoli var. typica (Pers.) Wint. Can. J. Bot. 45: 447-450.

Manners, J.M., Maclean, D.J., and Scott, K.J. 1982. Pathways of glucose assimilation in P. graminis f.sp. tritici. J. Gen. Microbiol. 128 (11): 2621-2630.

Manocha, M.S., and Shaw, M. 1966. The physiology of host-parasite relations. XVI. Fine structure of the nucleus in the rust-infected mesophyll cells of wheat. Can. J. Bot. 44(5): 669-673.

McKillican, M.E. 1960. A survey of amino acid content of uredospores of some races of wheat rust (Puccinia graminis). Can. J. Chem. 38: 244-247.

Meihle, B.R., and Lukezic, F.L. 1972. Studies on conidial germination and appressorium formation by Colletotrichum trifolii Bain and Essary. Can. J. Microbiol. 18: 1263-1269.

Mendgen, K., Lange, M., and Bretschneider, K. 1985. A quantitative estimation of the surface carbohydrates of infection structures of rust fungi with enzymes and lectins. Arch. Microbiol. 140: 307-311.

Miflin, B.J., and Lea, P.J. 1977. Amino acid metabolism. Annu. Rev. Plant Physiol. 28: 299-329.

Morris, D.R., and Fillingame, R.H. 1974. Regulation of amino acid decarboxylation. Ann. Rev. Biochem. 43: 303-325.

Otto, F. and Tsou, K.C. 1985. A comparative study of DAPI, DIPI, and Hoechst 33258 and 33342 as chromosomal DNA stains. Stain Tech. 60 (1): 7-11.

Plesosky-Vig, N., and Brambl, R. 1985. Heat shock response of Neurospora crassa: Protein synthesis and induced thermotolerance. J. Bacteriol. 162(3): 1083-1091.

Rajam, M.V., Weinstein, L.H., and Galston, A.W. 1985. Prevention of plant disease by specific inhibition of fungal polyamine biosynthesis. Proc. Nat'l. Acad. Sci. U.S.A. 82: 6874-6878.

Reisener, H.J. 1967. Untersuchungen uber den Aminosaure und Proteinstoffwechsel der Uredosporen des Weizenrostes. Arch. Mikrobiol. 55: 382-397.

Reisener, H.J., and Jager, K. 1966. The quantitative importance of some metabolic pathways in uredospores of Puccinia graminis var. tritici. Planta 72: 265-283.

Reisener, H., McConnell, W.B., and Ledingham, G.A. 1961. Studies on the metabolism of valerate-1-C^{14} by uredospores of wheat stem rust. Can. J. Biochem. Physiol. 39: 1559-1566.

Rines, H.W., French, R.C., and Daasch, L.W. 1974. Nonanal and 6-methyl-5-hepten-2-one: Endogenous germination stimulators of uredospores of P. graminis tritici and other rusts. J. Agr. Food Chem. 22: 96-100.

Saunders, S. and Youatt. 1983. Amino acids in the control of differentiation of sporangia in Allomyces macrogynus. Aust. J. Biol. Sci. 36: 435-443.

Savile, D.B.O. 1939. Nuclear structure and behaviour in species of the Uredinales. Am. J. Bot. 26: 585-609.

Schiff, J.A. and Hodson, R.C. 1973. The metabolism of sulphate. Ann. Rev. Plant Physiol. 24: 381-414.

Scott, K.J. 1972. Obligate parasitism by phytopathogenic fungi. Biol. Rev. 47: 537-572.

Scott, K.J. 1976. Growth of biotrophic parasites in axenic culture. In Physiological plant pathology, eds. R. Heitefuss, P.H. Williams. New York: Springer-Verlag.

Shaw, M. 1963. The physiology and host-parasite relations of the rusts. Ann. Rev. Phytopath. 1: 259-294.

Shaw, M. 1964. The physiology of rust uredospores. Phytopath. Z. 50: 159-180.

Shaw, M., and Colotelo, N. 1961. The physiology of host-parasite relations. VIII. The effect of stem rust on the nitrogen and amino acids in wheat leaves. Can. J. Bot. 39: 1351-1372.

Shaw, M., Boasson, R., and Scrubb, L. 1985. Effect of heat shock on protein synthesis in flax rust uredosporlings. Can. J. Bot. 63: 2069-2076.

Singh, H. and Sethi, I. 1982. Growth responses of race 222 of P. graminis f. sp. tritici in defined media. Can. J. Microbiol. 28: 486-492.

Staples, R.C. 1962. Initial products of acetate utilization by bean rust uredospores. Contrib. Boyce Thompson Inst. Plant Res. 21: 487-497.

Staples, R.C. 1965. Acid phosphatases from healthy and rust infected Pinto Bean leaves. II. Effect of removing apical meristem. Contrib. Boyce Thompson Inst. Plant Res. 23(4): 83-91.

Staples, R.C. 1968. Protein synthesis by uredospores of the bean rust fungus. Neth. J. Plant Path. 74: 25-36.

Staples, R.C. 1974. Synthesis of DNA during differentiation of bean rust uredospores. Physiol. Plant Path. 4: 415-424.

Staples, R.C., Syamananda, R., Kao, V., and Block, R.J. 1962. Comparative biochemistry of obligately parasitic and saprophytic fungi. II. Assimilation of ^{14}C-labelled substrates by germinating uredospores. Contrib. Boyce Thompson Inst. 21: 345-362.

Staples, R.C. and Stahmann, M.A. 1964. Changes in protein and several enzymes in susceptible bean leaves after infection by the bean rust fungus. Phytopathology 54: 760-764.

Staples, R.C and Wynn, W.K. 1965. The physiology of uredospores of the rust fungi. Botan. Gaz. 31: 537-564.

Staples, R.C. and Bedingian, D. 1967. Preparation of an amino acid incorporation system from uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. Plant Res. 23: 345-347.

Staples, R.C., Bedingian, D. and Williams, P.H. 1968. Evidence for polysomes in extracts of bean rust uredospores. Phytopathology 58: 151-154.

Staples, R.C., Grambow, H.J. and Hoch, H.C. 1982. Potassium ion induces bean rust germlings to develop infection structures (abstr. 221). Phytopathology 72: 957.

Staples, R.C. and Hoch, H.C. 1982. A possible role for microtubules and microfilaments in the induction of nuclear division in bean rust uredospore germlings. Exp. Mycol. 6: 293-302.

Staples, R.C., Grambow, H.J., Hoch, H.C. and Wynn, W.K. 1983a. Contact with membrane grooves induces wheat stem rust uredospore germlings to differentiate appressoria but not vesicles. Phytopathology 73 (10): 1436-1439.

Staples, R.C., Macko, V., Wynn, W.K. and Hoch, H.C. 1983b. Terminology to describe the differentiation response by germlings of fungal spores. Phytopathology 380.

Staples, R.C., Gross, D., Tiburzy, R., Hoch, H.C., Hassouna, S., and Webb, W.W. 1984. Changes in DNA content of nuclei in rust uredospore germlings during the start of differentiation. Exp. Mycol. 8: 245-255.

Stefanye, D. and Bromfield, K.R. 1965. Amino acid composition of uredospores of P. graminis tritici. Can. J. Bot. 43: 695-699.

Sziraki, I., Barna, B., Waziri, E., and Kiraly, Z. 1976. Effect of rust infection on the cytokinin level of wheat cultivars susceptible and resistant to P. graminis tritici. Acta Phytopath. Acad. Sci. Hung. 11: 155-160.

Tabor, C.W. 1981. Mutants of Sacchromyces cerevisiae deficient in polyamine biosynthesis: studies on the regulation of ODC. Medical Biology 59: 272-278.

VanSumere, C.F., VanSumere de Preter, C., Vining, L.C. and Ledingham G.A. 1957. Coumarins and phenolic acids in the uredospore of wheat stem rust. Can. J. Microbiol. 3: 847-862.

Von Broembsen, S.L., and Hadwiger, L.A. 1972. Characterization of disease resistance responses in certain gene-for-gene interactions between flax and Melampsora lini. Physiol. Plant Path. 2: 207-215

Walters, D.R. 1986. The effects of a polyamine biosynthesis inhibitor on infection of Vicia faba L. by the fungus Uromyces vicia-fabae (Pers.) Schroet. New Phytol. 104: 613-619.

Wanner, R.H., Forster, K., Mendgen, R.C. and Staples, R.C. 1985. Synthesis of differentiation-specific proteins in germlings of the wheat stem rust fungus after heat shock. Exp. Mycol. 9: 279-283.

Whitney, H.S., Shaw, M., and Naylor, J.M. 1962. The physiology of host-parasite relations. XII. A cytophotometric study of the distribution of DNA and RNA in rust-infected leaves. Can. J. Bot. 1533-1544.

Williams, P.G. 1971. A new perspective of the axenic culture of Puccinia graminis f.sp. tritici from uredospores. Phytopathology. 61: 994-1002.

Williams, P.G. 1984. Obligate parasitism and axenic culture. In The Cereal Rusts, vol. 1, 399-428. New York: Academic Press Inc.

Williams, P.G., Scott, K.J., and Kuhl, J.C. 1966. Vegetative growth of Puccinia graminis f.sp. tritici in vitro. Phytopathology. 56: 1418-1419.

Wilson, E.M. 1958. Aspartic and glutamic acids as self-inhibitors of uredospore germination. Phytopathology 48: 595-600.

Wisdom, C. 1977. Morphogenesis of the wheat stem rust uredospore. M.Sc. Thesis. University of British Columbia.

Wolf, G. 1982. Physiology and biochemistry of spore germination. In The Rust Fungi, eds. K.J Scott and A.K. Chakravorty, 151-178. New York: Academic Press.

Wynn, W.K., Staples, R.C., Strouse, B. and Gajdusek, C. 1966. Phsiology of uredospores during storage. Contrib. Boyce Thompson Inst. 23: 229-242.

Wynn, W.K., and Staples, R.C. 1981. Tropisms of fungi in host recognition. In: Plant Disease Control: Resistance and Susceptibility, eds. R.C. Staples, G.H. Toniessen, 45-69. New York: Wiley.

Yaniv, Z. and Staples, R.C. 1974. Ribosomal activity in urediospores germinating on membranes. Phytopathology 64: 1111-1114.

Youatt, J. 1986. Evidence of methionine biosynthesis in Allomyces macrogynus. Trans. Br. Mycol. Soc. 86 (4): 653-655.

Zadoks, J.C. 1968. Meterological factors involved in the dispersal of cereal rusts. In Proc. Regional training seminar on Agro-meterology, ed. A.J.W. Borghorst. Wageningen: 179-194.