A New Perspective of the Axenic Culture of Puccinia graminis f. sp. tritici from Uredospores

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ABSTRACT

Applying the Giemsa stain to nutrient agar cultures of *Puccinia graminis* f. sp. *tritici* shows that dikaryotic mycelia are formed only by uredospores which differentiate an infection structure. Saprophytic cultures also contain monokaryotic haploid mycelia. These mycelia originate from germ tubes which fail to differentiate. The mode of origin of haploid mycelia suggests that in normal differentiation the substomatal vesicle is the site of derepression of nuclear functions concerned with the metabolism of vegetative cells. Experiments demonstrate that the formation of infection structures in culture is affected by nutrients, heat shock, the time of collecting the inoculum, and genetic constitution. The results are discussed in relation to the new perspective they provide of the theory and practice of axenic rust culture. Phytopathology 61:994-1002.

Additional key words: wheat stem rust, cytology, morphogenesis.

The establishment of a vegetative mycelium of *Puccinia graminis* f. sp. *tritici* in wheat is preceded by the differentiation of an infection structure by a uredospore germ tube. Dickinson (7) and Chakravati (3) showed that the formation of an infection structure is an essential prelude to vegetative growth, even when the natural barrier to infection, the epidermis, is removed. Some of the factors affecting differentiation have been elucidated, and Allen (1) expressed the view that such factors might prove to be important in achieving the axenic culture of the wheat stem rust pathogen. However, Williams et al. (22), who succeeded in this objective, found that saprophytic mycelia grew from undifferentiated germ tubes as well as from those which had differentiated an infection structure (21). These authors therefore subscribed to the long established view that the culturing of rust fungi in vitro was a question of providing suitable nutrients. The observations reported in this paper show that my colleagues and I were mistakenly led to this conclusion by assuming that the mycelia of different origin are identical. Because of the fundamental way in which they differ, it now appears that the differentiation of an infection structure is just as essential for the establishment of the dikaryophase on nutrient agar as it is in nature. This paper describes a cytological study of axenic cultures of *P. graminis* f. sp. *tritici* demonstrating the importance of the infection structure. Experiments showing the effects of several factors on the formation of infection structures on nutrient agar are also reported.

MATERIALS AND METHODS.—*Organisms.*—Most of the experiments reported here used a new isolate of strain 126-Anz-6,7 of *Puccinia graminis* (Pers.) f. sp. *tritici* Eriks. & E. Henn. This isolate, bearing the Sydney University culture No. 70165, possesses the same virulence characteristics as the isolate (culture 334) used in the former investigation of axenic culture in the Department of Biochemistry at the University of Sydney. However, the saprophytic growth of culture 70165 differs in certain respects to that of culture 334 (M. J. Hartley & P. G. Williams, *unpublished data*).

Other strains used include 126-Anz-6,7 (culture 334), 21-Anz-1,2,3,7 (culture 70180), 111-E-2 (culture 56-L-1), and NR-2.

Production and collection of inoculum.—Uredospores for inoculation were collected from rust infections on seedling leaves of susceptible wheat (*Triticum aestivum* L. W2691). Seedlings were grown in a controlled environment cabinet at 21-23 C with a 16-hr photoperiod (5,000 ft-c), and uredospores were collected by tapping infected leaves held inside a sterile glass tube (13 × 88 mm). Contamination was minimized by collecting from uredosori which had been open for not more than 6-7 days. When it was necessary to incubate cultures for longer than 1 or 2 days, contaminants were removed with a cork-borer.

Inoculation and incubation of axenic cultures.—Freshly collected uredospores were suspended in 1-3 ml Freon 113 (trichlorotrifluoroethane) and sprayed onto 10 ml nutrient medium in plastic petri dishes (8.5 cm diameter) (16). The nutrient medium (pH 6.4) contained $NaNO_3$, 1 g; K_2HPO_4, 0.5 g; KCl, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 5 mg; trisodium citrate, 1.5 g; D-glucose, 30 g; peptone (Evans Medical Ltd., Speke, Liverpool, England), 5 g; Difco-Bacto agar (Difco Laboratories, Michigan), 15 g; and distilled water to 1 liter. Cultures were incubated at 23 C, and no precaution was taken to minimise evaporation. When required, a heat shock was given by transferring cultures 1 hr after seeding to a 30 C incubator. Two hr later, they were returned to 23 C. The incidence of infection structures was determined 24-30 hr after inoculation. At least 100 germinated spores were counted in microscope fields at ×50 magnification. The frequency (p) of sporelings that had formed an infection structure is expressed as a percentage of the total number of sporelings that had germinated without aborting (16). Confidence limits for p were obtained from published tables (9).

Staining procedure.—Growing fungal cultures on pieces of Cellophane overlying nutrient agar is a technique in common use for cytological work, and this

method is satisfactory for growing wheat stem rust cultures from mass inocula. However, the development of mycelia from sprayed inocula is poor on Cellophane, perhaps because the Cellophane interferes with mutual stimulation (16). It was, therefore, necessary to develop a method for staining nutrient agar cultures in toto. The following procedure was adapted from Colotelo & Grinchenko (6) and Hiratsuka & Maruyama (14). Pieces, about 1.5 cm², were cut from cultures on nutrient agar and mounted on microscope slides previously coated with 4% water agar. The cultures were fixed in OsO_4 vapor, dried and then hydrolysed in N HCl at 60 C for 6.5-7 min. Hydrolysis was terminated by transferring to 0.2 M potassium phosphate, pH 6.9. Thereafter, the slides were kept horizontal, as hydrolysis weakens the adhesion of the preparation to the slide. Following several changes of 0.02 M phosphate of the same pH, Giemsa stain (6) made from the dry powder (G. T. Gurr, London, England) was applied for 40-60 min with periodic, gentle agitation. Excess stain was removed by briefly rinsing in dilute buffer. The preparations were made permanent by allowing them to dry, and were mounted in immersion oil for observations.

RESULTS.—*Morphology and cytology of sporelings and hyphae.*—Sporelings in saprophytic cultures fall into two morphological classes distinguishable by the presence or absence of an infection structure (Fig. 1). Some individuals of each kind produce a saprophytic mycelium, but the number doing so has not been studied. The total number initiating a mycelium is affected by the thickness of the nutrient agar (16).

Williams et al. (22) attached no significance to the different modes of origin of mycelia. However, staining axenic cultures demonstrates that there is a fundamental difference in the nuclear condition of saprophytic hyphae, depending on whether they grow from a substomatal vesicle or from a germ tube. It appears that the substomatal vesicle is essential for the initiation of dikaryotic hyphae (Fig. 2). Figure 3 shows camera lucida drawings indicating nuclear distribution in three sporelings of different morphology observed in a culture incubated at constant temperature for 5 days. These examples were selected to demonstrate the nuclear distribution typical of sporelings of particular morphological types seen in many cultures. Nuclear distributions that occur rarely are so indicated.

A complex mycelium formed from an infection structure is always composed of binucleate cells (Fig. 3-A). Limited hyphal growth from the vesicle is usually associated with an abnormal nuclear condition; e.g., trinucleate cells. Hyphae growing from germ tubes are most frequently composed of uninucleate cells. Rarely, binucleate hyphae occur, but these have not been seen to form a mycelium. A sporeling giving rise to mainly uninucleate hyphae is shown in Fig. 3-B. The germ tube is divided into cells by septa formed at irregular intervals. The number of germ tube cells and their nuclear content are variable in sporelings of this type. Although uninucleate cells are most common, cells containing 0, 2, or 3 nuclei have been observed. Uninucleate hyphae

originate from uninucleate cells which, in some cases, are noticeably larger than other cells in the germ tube (Fig. 1-C).

Sporelings which consist of a simple germ tube and have produced no saprophytic hyphae (Fig. 1-D) generally lack septa and contain 2 nuclei only. However, the nuclear distribution in some simple germ tubes indicates that one nuclear division sometimes occurs and is followed by the laying down of 1 or 2 septa at random.

In some cultures, it is not uncommon to find sporelings which have produced mycelium from the germ tube and from the infection structure. An example of this condition is shown in Fig. 3-C. The germ tube of this sporeling is septate, in contrast to the nonseptate condition normally present in a sporeling which has differentiated an infection structure in culture (Fig. 3-A), on a membrane (7, 18), or on the epidermis of a host plant (2). In the example shown, uninucleate hyphae grew out at three points along the germ tube, and a binucleate hypha grew out at a fourth point. The substomatal vesicle, on the other hand, has produced only binucleate hyphae.

Occasionally, sporelings can be found to have differentiated only a substomatal vesicle (Fig. 1-B). Staining shows that the mycelium formed by such a sporeling is binucleate. Thus, the appressorium is not essential in the formation of dikaryotic hyphae.

Several examples have been found of another rare but significant condition. In these, a germ tube 3-5 times the length of the uredospore and containing 2 nuclei had grown out from one germ pore. In addition, an appressorium, an infection peg, and a substomatal vesicle had formed on a germ tube produced from a second germ pore. The infection structure was anucleate. The condition of such sporelings indicates that morphological differentiation is regulated differently to the cytological processes which normally accompany it.

Staining cultures of culture 334 of strain 126-Anz-6,7 and strain 21-Anz-1,2,3,7 showed a similar variety in the patterns of nuclear distribution to that described above. The association of binucleate mycelia with infection structures and uninucleate mycelia with germ tubes was also observed in cultures of these isolates. These observations indicate that the initiation of a binucleate mycelium requires the formation of at least a substomatal vesicle. Therefore, a knowledge of the factors affecting the differentiation of uredospore germ tubes will be valuable for studies of rust development in axenic culture. The effect of several factors on differentiation are described below.

The nature of uninucleate mycelia.—Chromosome counts have not yet been obtained to establish the ploidy of the uninucleate mycelia observed in axenic cultures. However, two kinds of indirect evidence indicate that the nuclei of these mycelia are haploid. Firstly, a haploid condition is indicated by the mode of origin. Observations of cultures stained at different times during the first 24 hr of incubation indicate that uninucleate cells are formed in germ tubes as a result of monokaryotization during germination of binucleate
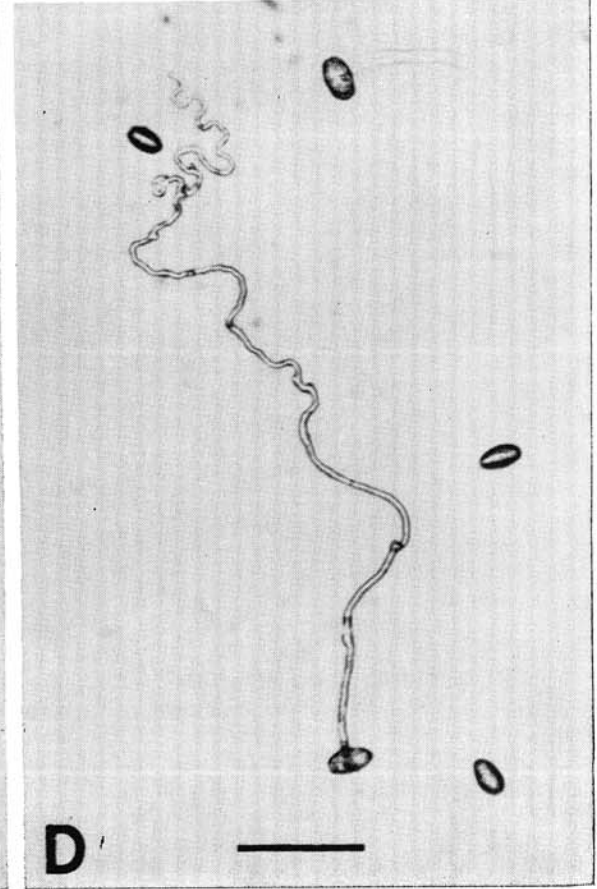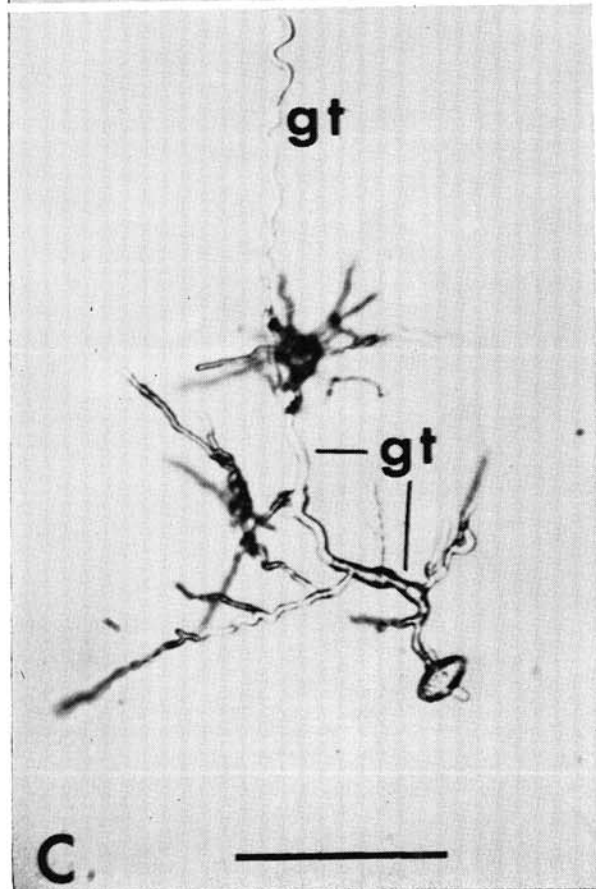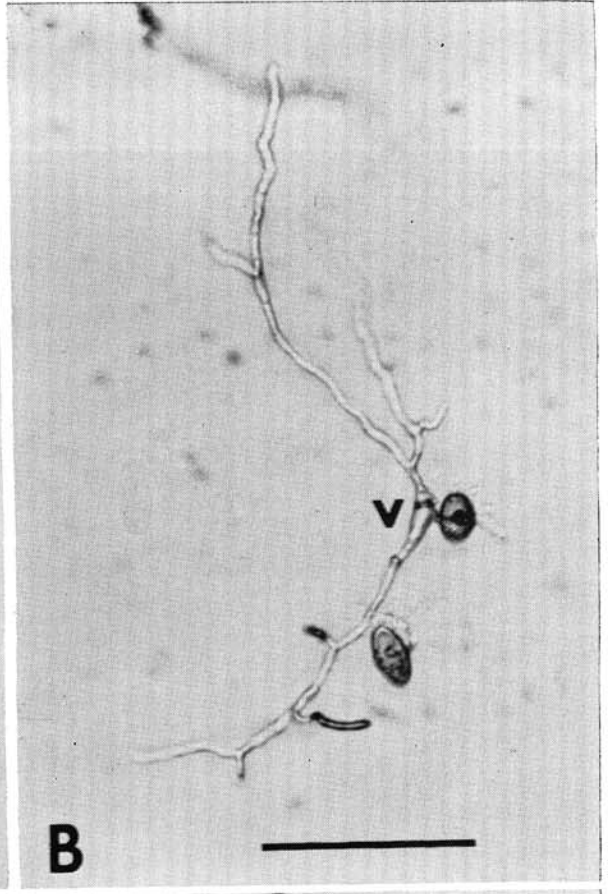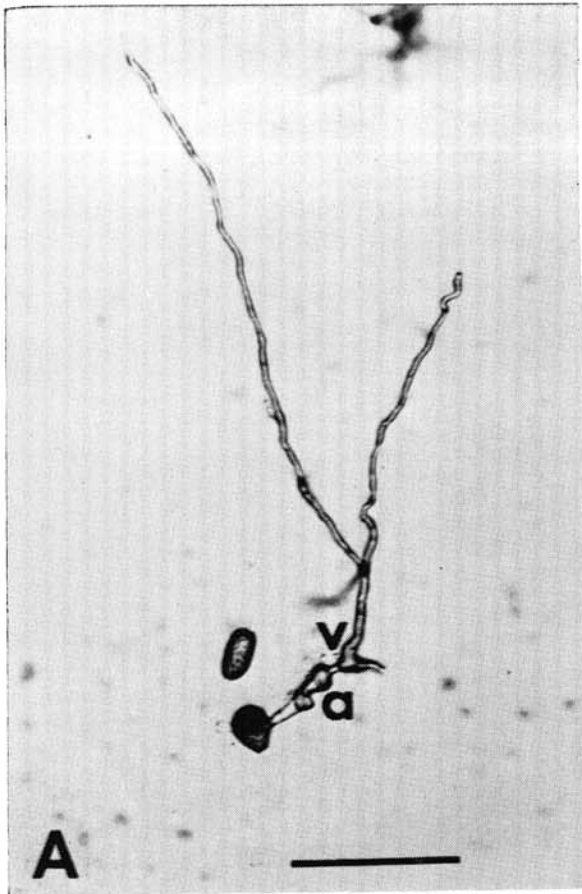
Fig. 2. Photomicrograph of a sporeling stained with HCl-Giemsa 72 hr after seeding. The germ tube (gt) has formed an appressorium (a) and substomatal vesicle (v). The hyphae that have grown from the vesicle contain conjugate pairs of nuclei (arrows). (×650)

uredospores. A similar phenomenon of monokaryotization has been reported in aeciospores of certain pine rusts germinated on a film of gelatin (4). Haploidy is also suggested by the observation that the occurrence, hyphal morphology, and growth characteristics of monokaryotic diploid mycelia (21) are distinctly different from those of the uninucleate mycelia in question. Monokaryotic diploid mycelia develop only after prolonged incubation of saprophytic cultures, are capable of indefinite propagation in subculture, and resemble dikaryotic mycelia in respect to hyphal diameter and frequency of branching. Monokaryotic haploid mycelia, on the other hand, develop early, do not grow in subculture, and are composed of hyphae that are narrower and more highly branched than those of monokaryotic and dikaryotic diploid mycelia [Fig. 4-A, B, C, D; and (21)].

The possibility that saprophytic cultures contain monokaryotic haploid as well as dikaryotic mycelia is significant for the interpretation of some recent experi-

ments on axenic culture of *P. graminis* f. sp. *tritici*. For example, the initiation and growth of saprophytic mycelia are reported to be variously affected by inoculum density and the addition of diverse compounds to the nutrient medium (5, 16). A reinvestigation of these variables is called for to distinguish between effects on morphogenesis and on the growth of monokaryotic and dikaryotic mycelia. The possibility of distinguishing between these mycelia by hyphal morphology may be useful in these investigations.

Factors affecting the formation of infection structures.—1) *Nutrients and heat shock.*—The effect of nutrients and heat shock on differentiation by *P. graminis* f. sp. *tritici* race 126-6,7 uredospores is shown in Table 1. Infection structures were formed rarely on water agar, and their frequency was not significantly increased by a heat shock. On nutrient agar, about one-sixth of the germinated uredospores formed an infection structure, and this fraction was increased to three quarters as a result of heat shock. This experiment con-

Fig. 1. Variation in sporeling morphology and origin of saprophytic hyphae. **A)** A sporeling which has differentiated a complete infection structure, including an appressorium (a) and substomatal vesicle (v). A branched hypha has grown from one lobe of the vesicle. **B)** A sporeling which has differentiated only a substomatal vesicle. Branched hyphae have grown from both lobes of the vesicle (v). **C)** A sporeling which has not differentiated an infection structure. Branched hyphae have emerged from several places on the germ tube (gt). **D)** A sporeling which has neither differentiated an infection structure nor produced saprophytic hyphae. Scale line = 0.1 mm unstained.
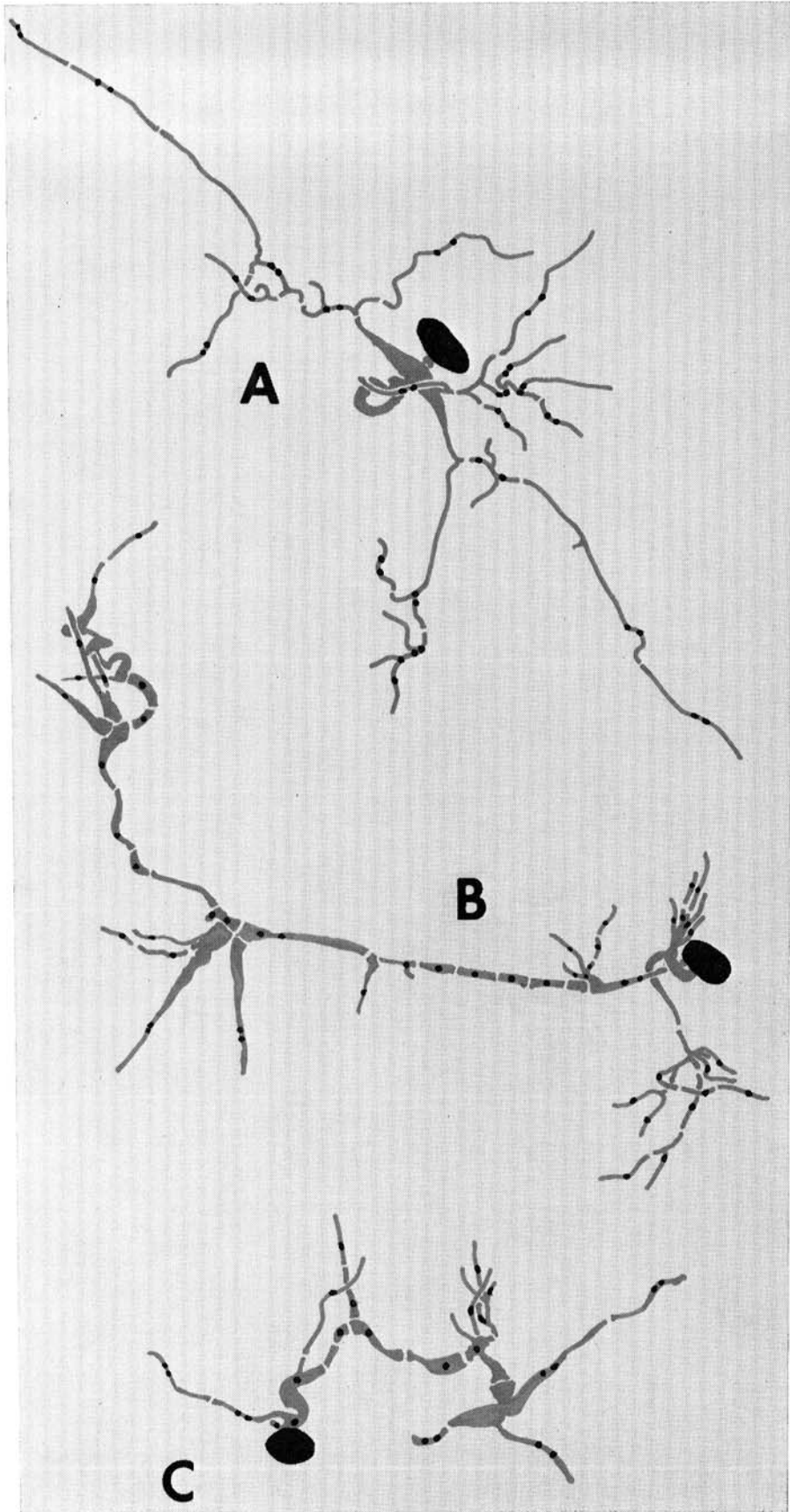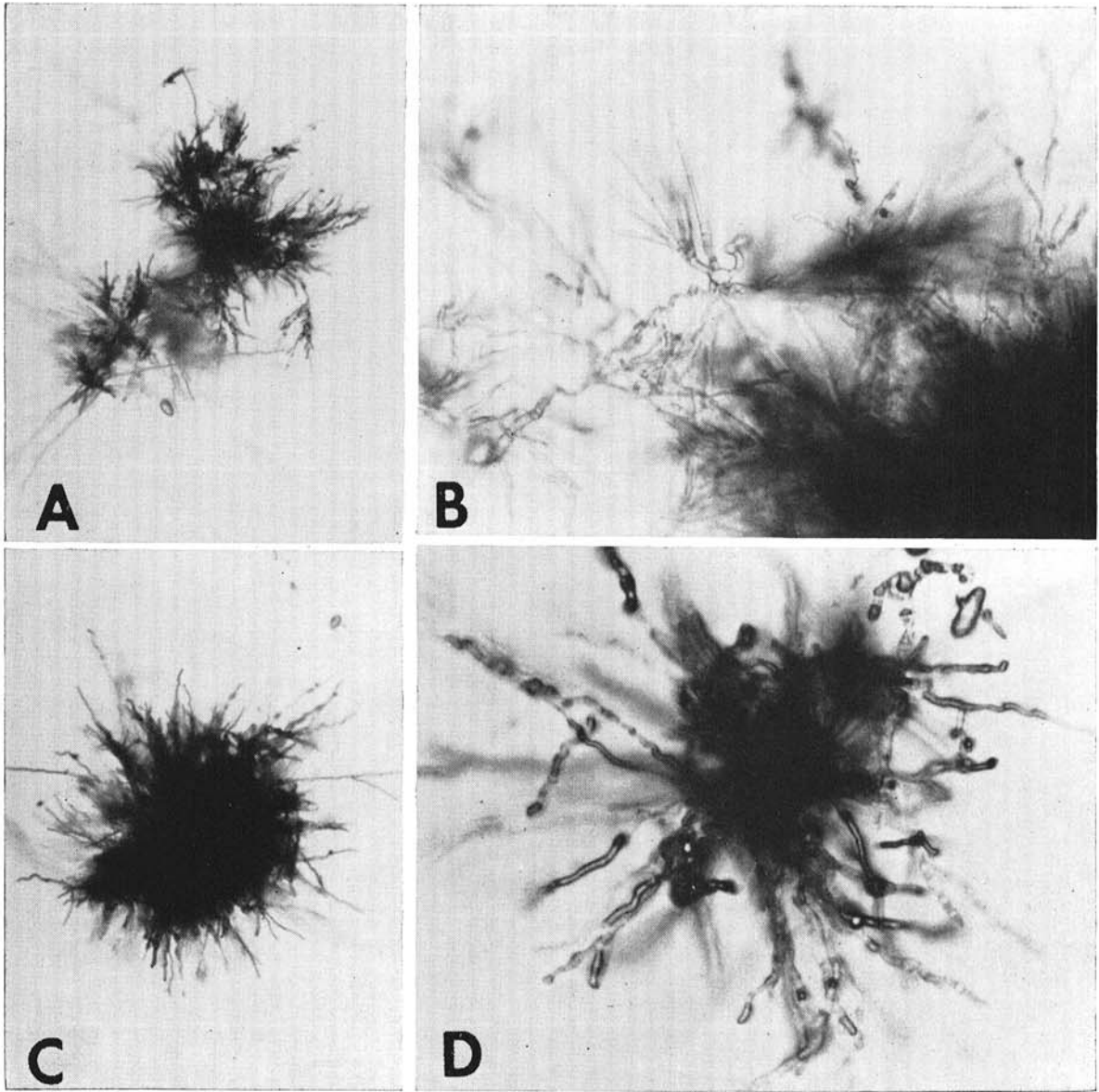
Fig. 4. A, B) Monokaryotic haploid and **C, D)** dikaryotic mycelia. (A, C, ×80; B, D, ×230)

firms that nutrients stimulate differentiation on agar (13, 15, 19), and indicates that the nutrient stimulus is more effective when a heat shock (12, 17) is applied. The effect on differentiation of raising the temperature of incubation has not been investigated fully, but experiments indicate that the maximum response to a 2-hr period at 30 C is obtained when incubation at 30 C is begun between 1 and 3 hr after seeding (M. J. Hartley,

unpublished data). The data in Table 1 also show that in the range 100-1,000 sporelings/cm² differentiation is not affected by the density of seeding.

The results of preliminary experiments in this laboratory confirm the earlier observation of Naito et al. (19) on *P. coronata* that no single constituent of the nutrient medium is wholly responsible for eliciting differentiation of germ tubes. In one experiment, no in-

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Fig. 3. Camera lucida drawings showing nuclear distribution in sporelings and associated mycelia. **A)** A sporeling which has differentiated an infection structure. The mycelia which have grown from the substomatal vesicle are composed of binucleate cells. **B)** A sporeling which has not differentiated an infection structure but has produced hyphae at several places on the germ tube. Most of the hyphae are composed of uninucleate cells. **C)** A sporeling which has differentiated an infection structure but has produced hyphae from the germ tube as well as from the vesicle. The latter hyphae are binucleate and most of the former are uninucleate. (×350)

TABLE 1. The effect of nutrients and a heat shock on the formation of infection structures by uredospores seeded on agar at high and low density

Mean density of germinated spores	Incubation conditions	Composition of agar	
		Water	Nutrient
		% Infection structures[a]	
1,000/cm^2	Constant temp	0 (0-1.8)	16 (12-22)
	Heat shock	2 (0.5-5.0)	72 (68-80)
100/cm^2	Constant temp	1 (0.1-3.6)	11 (7-16)
	Heat shock	0 (0-2.2)	69 (63-76)

[a] Figures in parentheses indicate the 95% confidence interval.

fection structures were produced on agar containing the mixture of minerals and sodium citrate (pH 6.4), 1% of germinated uredospores differentiated on glucose agar buffered with 0.001 M potassium phosphate, and 5% on peptone agar, pH 6.4. All the combinations of these ingredients in pairs increased differentiation to 16%, but 35% differentiation was obtained on the complete mixture. These results indicate that the response to nutrients is not simply determined and requires further, systematic study.

2) *Time of collecting the inoculum.*—Inocula collected at the same time each day, but on different days during sporulation varied in ability to form infection structures (Table 2). The ability to differentiate at constant temperature was greatest in samples of spores collected on the 2nd day after the uredosori opened, and declined progressively in the samples collected from the same sori on the 4th and 8th day. The data in Table 2 also show that although the incidence of infection structures decreased with increasing age of the uredosori, the formation of infection structures in response to a heat shock remained at a high level (about 70%). This result suggests that as the uredosori age, an increasing proportion of the uredospores released lack the necessary sensitivity to the nutrient stimulus to differentiate without a heat shock. To understand this effect, information is needed about the sequence of production, maturation, and release of uredospores.

3) *Genetic factors.*—Strains of *P. graminis* f. sp. *tritici* differ in ability to form infection structures on nutrient agar. In the experiment shown in Table 3, wheat seedlings were inoculated with four strains of *P.*

TABLE 2. The formation of infection structures by uredospores collected at different times during sporulation

Incubation conditions	Days after the opening of uredosori		
	2	4	8
	% Infection structures[a]		
Constant temp	52 (46-56)	38 (34-43)	24 (22-32)
Heat shock	78 (73-81)	80 (74-82)	64 (59-68)

[a] Figures in parentheses indicate the 95% confidence interval.

TABLE 3. The frequency of infection structures in cultures of different strains of *Puccinia graminis* f. sp. *tritici*. Inocula collected at different times during sporulation

Strain	Incubation conditions	Days after opening of uredosori		
		2	4	8
		% Infection structures		
126-Anz-6,7 (culture 70165)	Constant temp	52	36	33
	Heat shock	82	78	83
21-Anz-1,2,3,7	Constant temp	39	26	2
	Heat shock	85	86	87
111-E-2	Constant temp	0	0	
	Heat shock	75	77	
NR-2	Constant temp	18	8	
	Heat shock	64	42	

graminis f. sp. *tritici,* and inocula were collected at the indicated times and tested for infection structure formation on nutrient agar. The inocula of 126-6,7 and 21-1,2,3,7 were generally superior in differentiating infection structures at constant temperature to the inocula of 111-E-2 and NR-2. The latter strains took longer to develop uredia than the former, and there was no tendency for the first collected inocula of 111-E-2 and NR-2 to give a high incidence of infection structures. Differentiation in all the strains tested was increased by a heat shock. This treatment was especially beneficial in the case of 111-E-2. The ability of strains 111-E-2 and NR-2 to form infection structures appears to be particularly weak. Since differentiation is critical for the initiation of dikaryotic mycelia, this observation may help to explain why saprophytic growth from mass inocula of these strains is so poor (16).

DISCUSSION.—The finding that the origin of dikaryotic mycelia in axenic cultures parallels the situation in nature vindicates the approach of Dickinson (7, 8), Allen (1) and others who sought an answer to the problem of axenic culture in the process of germ tube differentiation. In view of the common belief that rust development in vitro is a question of nutrition only, it is perhaps ironic that the nutrients used to culture *P. graminis* f. sp. *tritici* are now seen to serve two essential functions: eliciting morphogenetic responses and supporting the growth of saprophytic hyphae. How well the present mixture of nutrients serves each of these purposes is a matter for further study; nevertheless, it is clear that the value of this medium for axenic culture depends, in the first instance, on its ability to elicit expression of the morphogenetic potential of the inoculum.

The variation in morphogenetic potential observed among individual uredospores and between successive inocula indicates that germ tube development is, in part, endogenously regulated. Allen (1) demonstrated that wheat stem rust uredospores release a volatile material which stimulates formation of infection structures when uredospores germinate on water or dilute buffer. At present there is no evidence that Allen's stimulator also affects germ tube development on nu-

trient agar. However, the variation reported here is consistent with the activity of such a volatile material during the initial stages of incubation of axenic cultures. Whether or not substances of this kind are involved, the results presented here indicate that the ability of uredospores to differentiate, and therefore, to initiate saprophytic mycelia is determined by processes in the uredosorus. The nature of the processes is such as to cause variation in the morphogenetic potential of inocula collected at different times in the sporulation phase of leaf infections. Variation from this source, affecting the condition of the inoculum at the time of seeding, may have contributed to the unpredictable behavior of *P. graminis* f. sp. *tritici* inocula encountered in studies at the University of Sydney (16). Controlled experiments on rust nutrition were finally abandoned there for want of inocula which would reproducibly form mycelia on a standard nutrient medium. Future experiments of this kind may benefit from an enquiry into the causes of variation in morphogenetic potential of uredospore inocula.

Evidence was obtained that strains of *P. graminis* f. sp. *tritici,* distinguishable by virulence characteristics, also differ in ability to form infection structures. The ability to differentiate is important in the production of saprophytic mycelia, and genetically determined differences in this ability might be manifest as differences in the ability of strains to grow in axenic culture. Such a correspondence has been noted already with respect to two strains which grow poorly. Strain 126-Anz-6,7, on the other hand, differentiates infection structures relatively freely, and this attribute may well have contributed to its successful culture in 1966.

Cytological studies of germ tube development (2, 7, 18) show that the differentiation of an infection structure is accomplished by a coordinated sequence of morphological and cytological events. Dickinson (7) proposed that differentiation on artificial membranes is triggered by a contact stimulus, and that a stimulus of optimum intensity is necessary for the sequence to proceed normally to its completion. Deviations from the normal pattern of development, he suggested, were the result of stimuli below or above the optimum level of intensity. However, the kinds of abnormal development which Dickinson observed and are reported here would also result if the stimulus to differentiate were uniform but the capacity of individual uredospores to respond were variable. This alternative interpretation is supported by the observation that the incidence of abnormal development varies in different inocula seeded on the same batch of nutrient agar. It is, furthermore, consistent with the possibility that germ tube morphogenesis is regulated by volatile or unstable endogenous compounds. It is not assumed that the stimulus on agar and on membranes is the same. This interpretation offers new insights into the process of differentiation relevant to studies of germ tube development in nature and axenic culture.

Morphological differentiation and cytological differentiation appear to be regulated independently. If, as is proposed, the capacity to differentiate is a variable attribute, then variation in the level of competence with respect to either or both of these components of differentiation is possible. A further dimension of variation may be introduced by variable coordination of the morphological and cytological sequences. Within the framework of these contingencies, the following interpretation can be given of the diverse forms of development observed in axenic cultures. One kind of uredospore is fully competent to carry out both the morphological and cytological steps in differentiation. Some uredospores of this type produce infection structures and normal dikaryotic hyphae. Others, in which coordination is defective, also initiate hyphae in the germ tube. A second kind of uredospore is deficient in either morphological or cytological potential. A uredospore deficient cytologically succeeds in differentiating an infection structure, but fails to produce normal dikaryotic hyphae. A spore deficient morphologically forms only a germ tube in which unregulated septation and nuclear division occur, and some of the cells so formed give rise to hyphae. A third kind of uredospore, deficient to a greater or lesser extent in both abilities, produces a germ tube in which septation and nuclear division is limited or absent. These germ tubes do not produce saprophytic hyphae. It is for future research to determine whether these forms of abnormal development are significant in nature. Furthermore, the concept of the morphogenetic potential of uredospores suggested here deserves consideration in other rust researches, in addition to those on axenic culture.

The different mode of origin of mono- and dikaryotic mycelia suggests that differentiation is accompanied by a cytological event whose nature has not previously been appreciated. This event, I suggest, is the derepression of nuclear genes which regulate the metabolism of vegetative cells. In fully competent uredospores, this event takes place when the daughters of the uredospore nuclei reach the substomatal vesicle, leading to the formation of a dikaryotic mycelium. Uredospores lacking adequate potential for morphological differentiation, as suggested above, undergo cytological differentiation involving septation and nuclear divisions. Some of the cells so formed appear to assume the function of the substomatal vesicle. These cells may be termed pseudo-vesicles. When a cell becomes a pseudo-vesicle, the vegetative functions of the nucleus or nuclei it contains are derepressed, and a hypha is initiated. Since germ tube cells are mainly uninucleate, most of the hyphae initiated from pseudo-vesicles are uninucleate. The growth of binucleate hyphae which sometimes emerge from germ tubes is always limited. This may signify that the nuclei in these hyphae are of the same mating type, a combination which might be expected to be ineffective in a heterothallic fungus such as *P. graminis*. The failure of extensive dikaryotic mycelia to grow from germ tubes indicates either that complementary pairs of nuclei rarely occur in germ tube cells or that such pairs infrequently come to lie in cells which become pseudo-vesicles.

The proposed function of the substomatal vesicle

accords with Dickinson's view (7) that the differences in infectivity and permeability between germ tubes and infection hyphae (8) derive from events occurring in the vesicle. A recent report that nucleoli appear during the differentiation of infection structures (11) further supports the idea that expression of the uredospore genome is incomplete during the early stages of germ tube development. Studies of RNA synthesis (20) and the effects of inhibitors (10) indicate that the activity of template RNA and synthesis of new kinds of protein are stimulated when germinating uredospores are induced to form infection structures. For these studies, uredospores were germinated on membranes floated on water. Similar experiments using a nutrient solution instead of water may yield direct evidence that the vegetative state is inaugurated in the substomatal vesicle.

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