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Controlled Infection by *Puccinia graminis* f. sp. *tritici* under Artificial Conditions

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I. Introduction

Most investigations of the cereal rusts require dependable methods for producing plant infection under artificial conditions. During natural epidemics, the explosive spread of rust infection on cereals suggests that infection occurs easily on a susceptible host. In my experience with *Puccinia graminis* E. Henn., however, reproducible control of the amount of infection has been difficult to achieve. This chapter will summarize my observations and insights acquired during 26 years at the Cereal Rust Laboratory into the infection process initiated by urediospores of the wheat stem rust pathogen.

II. Production of Inoculum

The dependable production of pure, viable inoculum is influenced by the host genotype and environmental conditions. The host cultivar, cultural conditions, isolation procedures to prevent contamination, and method of harvesting spores can affect the quantity and quality of inoculum produced.

A. HOST SELECTION AND STATUS

Obviously, a suitable host cultivar for inoculum production must be susceptible to the rust cultures under investigation. The ideal cultivar for increasing rust would be a universal suscept to all genotypes of the rust pathogen. By definition, a universal suscept for a pathogen has no specific genes for resistance to that pathogen. At the Cereal Rust Laboratory, however, a universal suscept for wheat stem rust has not been found. The wheat cultivars Little Club, Baart, McNair 701, and W2691 are susceptible to most genotypes of the wheat stem rust fungus and frequently are used to propagate this rust pathogen. The soft white wheat cultivars Little Club and Baart possess a specific gene for resistance, *SrLc*, for which the corresponding gene for avirulence occurs with a low frequency in the North American population of *P. graminis* f. sp. *tritici* (Roelfs and McVey, 1979). The soft red wheat cultivar McNair 701 is used at the Cereal Rust Laboratory as the increase host for propagating rust from collections of wheat stem rust in the annual race survey. Several cultures of *P. graminis* f. sp. *tritici* isolated from aecia on barberry, however, are avirulent on McNair 701, and these

indicate the presence of a resistance gene, *SrMcN* (Roelfs and McVey, 1979). Watson and Luig (1963) tried to develop a universal suscept by selection of suitable progenies from the cross Little Club//Gabo*3/ Charter. The selected line, W2691, has been susceptible to all North American isolates of *P. graminis* f. sp. *tritici* tested. It is resistant, however, to certain cultures of *P. graminis* f. sp. *secalis* that can attack some wheat cultivars (Roelfs and McVey, 1979). Thus W2691 would not be a satisfactory host for increasing field collections of rusted wheat infected by such genotypes. Thus cultivars that appear to be universally susceptible are likely to possess unrevealed resistances simply because of the lack of a test with rust cultures that possess the appropriate gene for avirulence.

Good yields of urediospores are produced by erect, long-lived, sturdy seedling leaves, whereas spores are difficult to harvest from recumbent, elongated, or flaccid leaves. The growth habit of seedling leaves is influenced by genotype and environment. Under a single set of environmental conditions, the length, width, and duration of the erect status of the first seedling leaf varies among wheat cultivars. Furthermore, some wheats have a genetic trait for premature senescence of seedling leaves exposed to stress from heat, drought, or high numbers of infections. Regulation of environmental conditions can alter the growth habit of the first leaf in wheat seedlings. Low temperatures (15°–20°C) and long light periods of high intensity favor the development of short, broad, sturdy leaves, whereas high temperatures (25°–30°C) and short light periods of low intensity favor the development of elongated, slender, weak leaves.

Another important consideration in selecting a host cultivar for propagating rust is a source of vigorous, healthy seed. Often, a cultivar highly susceptible to the rust pathogen is unavailable commercially, and rust workers must produce their own seed. Thus the selected cultivar should be agronomically adapted to the growing conditions where it will be increased. Furthermore, it is important that the seed be free from seedborne pathogens that cause seed rots, damping off, and seedling blights. These diseases debilitate or kill the host plants, and spores of the unwanted pathogen may contaminate the harvested rust spores.

B. ENVIRONMENTAL CONDITIONS

The prevailing environmental conditions largely determine the longevity and productivity of increase cultures of rust. Uredial infections

of *P. graminis* grow indeterminately as long as vigorous chlorenchyma cells are accessible to the parasitic hyphae. Thus the longevity of an increase culture is dependent on that of the host leaves. First leaves of wheat live about 30 to 35 days after emergence at 18° to 21°C with 12 hr of sunlight per day in a glasshouse. Primary uredia produce spores continuously for about 10 days, with maximum production from 4 to 8 days after the uredium erupts through the host epidermis. Secondary uredia appear around the senescing primary uredium and produce additional spores as long as the host leaf remains photosynthetically active. Prabhu and Wallin (1971) found that spores were released for 16 days from a single uredium on a seedling leaf at 24°C with a 12-hr light period of 8600 lux.

Under my conditions, 18°–21°C was the most suitable temperature range for producing inoculum of the wheat stem rust pathogen. Sporulation is greater at 24° and 28°C than at 18°C (Prabhu and Wallin, 1971), but high temperatures shorten the longevity of the first leaf and favor the development of other pathogens. Similarly, moderate moisture conditions prolong inoculum production. When atmospheric and soil moisture is excessive, guttation drops form and wet leaf surfaces, favoring infection by contaminants including rust. Continuous exposure to such conditions causes some urediospores to germinate within uredia, which reduces inoculum quality. High temperatures and humidities often occur in small, enclosed isolation chambers used in glasshouses to protect rust cultures from contamination, and these conditions can kill the spores *in situ*. Filtered ventilation can prevent excessively high temperatures and humidity (Emge *et al.*, 1970). Inoculum production is curtailed by drought, which unduly stresses infected plants because of high transpiration rates through ruptures in the epidermis.

Duration and intensity of light are essential factors in the vigor and longevity of infected leaves and the production of inoculum. In Minnesota during winter, day length is too short and the intensity too low in glasshouses to support adequate spore production by wheat stem rust. About 8 hr of supplementary light from fluorescent lamps in open frames without reflectors mounted 10–15 cm above the tips of the wheat seedlings are used to supplement and compensate for the inadequate natural light. This provides about 10,000 lux of supplemental light at the leaf tips.

Maleic hydrazide is used routinely at the Cereal Rust Laboratory to control the growth of wheat and oat seedlings used to propagate stem rust in the glasshouse. This compound, applied to the soil surface when coleoptiles emerge, permits the first foliar leaf to develop and

remain erect, suppresses growth of secondary leaves, and prolongs sporulation. Under our conditions the effective dose in 50 ml of water added to the soil in a 10-cm plastic pot is 5 and 10 mg in winter and summer, respectively.

C. PURITY

Perpetuating a pure culture of cereal rusts through successive transfer generations in the presence of other rust cultures is difficult. Urediospores become airborne so readily that precautions are needed to prevent the deposition of contaminating spores on the host before inoculation, during the inoculation procedures, and throughout incubation and sporulation of the culture. Single uredial transfers aid in maintaining a pure culture of rust. At the Cereal Rust Laboratory we select a vigorous plant with a single, well-developed isolated uredium near a leaf base, and we remove the leaf blade above the selected uredium and other infected plants in the pot. The isolated plant and uredium are washed to remove spores, and the plant is exposed to dew for a 10- to 12-hr dark period. This dew period renders the remaining spores inocuous by inducing their germination. After the dew period, the infected plant is held in an isolation booth for 48 hr while the uredium produces a new crop of spores. These spores are collected and used as inoculum for the next transfer generation.

Single-spore isolation is the surest procedure for establishing a pure rust culture. Urediospores adhere poorly to glass, moderately well to animal hairs, and strongly to plant cuticle. If dry spores are dusted lightly on a glass slide, individual spores can be located under a stereoscopic microscope at about ×60 and picked up manually on the tip of a short animal hair that has been cemented to a handle. Hairs that taper to a fine point such as guard hairs from a straight-haired dog are best. The hair is inspected microscopically to ensure that only a single urediospore is present. Then the spore is touched to a leaf surface while observing microscopically to be sure that it adheres to the cuticle when the hair is removed. Depositing the spore near an India ink mark on the leaf conveniently locates the site where the infection should appear. Acetone or 95% ethanol removes unwanted spores from the hair.

After exposure to a suitable dew period (Section VII) for infection and incubation in a rust-free environment for 4 days, the plants are inspected daily for the appearance of the fleck stage of infection. Infected plants are transferred to individual isolation chambers to permit the

uredium to develop as a source of a pure line. Often with *P. graminis*, infection success is less than 10%, so 30 to 40 single-spore isolations are needed to ensure the development of at least two uredia. Inoculum from each uredium is increased and tested individually against appropriate differential cultivars to establish the identity of the pure cultures. After a pure culture is established, propagation in glass or plastic isolation chambers in a glasshouse will maintain purity.

Airborne, foliar pathogens also are troublesome contaminants, and *Erysiphe graminis* DC. is the most common one in cereal rust cultures. Sulfur controls powdery mildew readily, but it cannot be applied directly to plants that will be inoculated or used as sources of rust inoculum, because of its fungicidal action on the rust spores. Vapor from sulfur placed on steam pipes controls the spread of powdery mildew and can inhibit active infections with no apparent effect on rust development or urediospore viability. In glasshouses heated with forced air, the sulfur is placed in glass dishes on strategically located electric hot plates. Care must be taken to heat the sulfur sufficiently for sublimation and not oxidation (96°–119°C), because sulfur dioxide can injure plants. Another effective fungicide is ethirimol (Millstem), a systemic fungicide specifically effective against *E. graminis*. When applied as 50 ml of a 40-ppm solution to the soil surface in a 10-cm pot at planting, it prevents the development of mildew for 4 to 5 weeks in cultures of *P. graminis* f. sp. *tritici* or *P. recondita* Rob. ex Desm., without any apparent effect on the rust pathogens (Rowell, 1972). A useful method for inactivating oidia of *E. graminis* present in urediospore inoculum is to disperse the inoculum in an oil carrier (Section IV,C), because oidia in oil do not infect susceptible hosts.

Helminthosporium sativum is another common foliar pathogen of small grains that can contaminate rust cultures and can affect urediospore germination, host penetration, and the development of infections (Stewart and Hill, 1965). Because *H. sativum* is seedborne, this contaminant can be avoided by using only clean, healthy seed. A contaminated rust culture can be purified of this fungus by the single-uredial transfer method.

Occasionally, *Darluca filum* (Biv.-Bern.) Cast. occurs as a hyperparasite of rust fungi. Use of spores from a single, healthy uredium for transfer inoculum eliminates this contaminant.

P. recondita is a refractory contaminant of cultures of *P. graminis* f. sp. *triitici*. Once established in a culture, it will predominate in one or two transfer generations under artificial conditions, because a greater percentage of its urediospores successfully infect the host than those of

the stem rust pathogen. Most leaf rust contamination of cultures of *P. graminis* f. sp. *tritici* can be avoided by using a host resistant to the former and susceptible to the latter pathogen such as McNair 701, a winter wheat cultivar that possesses the resistance conditioned by *LR9*. Use of triazbutyl (Indar), a fungicide specifically effective only against *P. recondita*, also prevents leaf rust contamination of wheat stem rust cultures. This fungicide is a good protectant but a weak eradicant, so it is most effective when plants are treated prior to infection (Rowell, 1972; see chapter by Rowell, Vol. II). Application of 50 ml of a 2-ppm solution to the surface of the soil in a 10-cm pot at least 2 days before plants are inoculated prevents leaf rust.

D. HARVEST

When a rust culture is sporulating abundantly, the spores can be collected about every other day. Again, precautions against contamination must be observed. The collecting equipment and the operator should be clean and free of any viable spores. Spores should be collected as early as feasible in the workday as soon as the infected plants are dry and free of any guttation moisture. Cyclone collectors (Tervet *et al.*, 1951) have been designed in a variety of sizes from microcollectors that harvest the spores from a single uredium (Browder, 1971), to macrocollectors that will collect gram quantities of spores from massed infected plants. These units are easily sterilized between collections and are the best means of collecting spores free from contamination. Masses of spores are easily dislodged from uredia when the leaves are shaken over an open collection dish, but this procedure also releases many airborne spores, which can contaminate other cultures in the work area. Harvested spores often contain bits of leaf tissues, aphids, and soil particles that can be removed by passage through a 60-mesh soil sieve.

III. Storage of Inoculum

A. FACTORS AFFECTING UREDIOSPORE LONGEVITY

The individual rust urediospore is a vegetative spore without a true dormancy. It remains in the resting state only in the absence of conditions favorable for germination. Although germination inhibitors pro-

duced naturally by the cereal rusts are sufficiently concentrated to inhibit germination in masses of urediospores as in uredia, these substances appear to have no effect on dispersed spores in contact with water, presumably resulting from dilution below the effective dose (Tollenaar and Houston, 1966). At room temperatures and moderate relative humidities, spores of *P. graminis* remain viable for about 4 to 6 weeks. Vacuum drying or freezing at ultralow temperatures are dependable methods for long-term storage of viable urediospores. Storage conditions, however, affect urediospore form, moisture content, germinability, semipermeability, and respiration.

1. *Morphological Effects*

Germination can be a misleading indicator of urediospore viability after storage, because the percentage of urediospores that will germinate varies with the method used (see Section IV,D). In my experience, placing urediospores in an oil drop on water has been the most reliable germination test, because it minimizes rehydration injury (see Section IV,D,4). Dried urediospores of *P. graminis* invariably are shriveled. The spore is flattened along the long axis and has a pronounced indentation at right angles to this axis across the flattened surface over the equatorial pores. The wall and protoplast of such dried spores rehydrate at differential rates on direct contact with water (Rowell, 1956), and this differential rehydration can injure the spore.

The differential hydration of wall and protoplast is readily observed with a microscope by watching the structural changes in dried spores on a glass slide as water is allowed to flow under the coverslip. The wall immediately expands to its original shape on contact with water, but the protoplast at first remains contracted in a granular central mass separated from the wall, and then most of them gradually swell to fill the wall volume in 20 to 60 min. When water is brought into contact with a drop of oil containing dispersed urediospores under a coverslip, the spores initially remain shriveled and the oil has no observable effect. Gradually over 20 to 60 min, the wall and protoplast of spores in the oil drop expand simultaneously to the original shape, and rarely is a spore observed in which the protoplast remains contracted. In this type of experiment, urediospores germinate poorly under glass coverslips, but germination does occur if the observations are made on dried urediospores covered with a small square of 0.013-mm polyethylene film, which is permeable to oxygen and carbon dioxide. Under the film, only a few of the expanded spores in direct contact with water germinate, whereas most of the expanded spores in the oil droplet germinate.

Thus the oil apparently reduces injury by preventing the sudden separation of the wall from the plasmalemma.

2. *Moisture Changes*

The moisture content of freshly collected spores will vary with the environmental conditions. Moisture content of urediospores cannot be determined precisely, because a constant weight is not attained in a drying oven. After a large weight loss in the first 24 hr, weight continues to decrease slowly with further drying. Conventional determination of spore moisture indicated that water content was linearly proportional (from about 4 to 20% water) to atmospheric relative humidity (from 0 to 80% RH), but abruptly increased to about 30% water at 92% RH (J. B. Rowell, unpublished data).

Urediospore germinability varies with time and humidity during storage. Dispersed spores stored at 20% RH have had the best overall survival and least rehydration injury (Table I). Differential rates of germination on water and in oil on water agar (Table I) show that rehydration injury is most severe at 0% RH, but unaccountably a similar, unexplained injury occurs after prolonged storage at 66% and high-

Table I

Effect of Time and Relative Humidity (RH) during Storage on Germinability of Dispersed Urediospores of *Puccinia graminis* f. sp. *tritici*[a,b]

Relative humidity (%)	Germination medium	Germination after indicated days of storage (%)		
		3	7	10
0	Water	22	10	0
20	Water	78	69	61
66	Water	85	15	4
84	Water	87	28	3
92	Water	93	55	0
0	Oil on agar	73	18	32
20	Oil on agar	82	70	63
66	Oil on agar	100	91	82
84	Oil on agar	97	69	12
92	Oil on agar	97	75	0

[a] J. B. Rowell, unpublished data.

[b] Dispersed spores on small squares of polyethylene film were exposed to indicated RH in sealed chambers, and germinability was determined by inverting the squares so the spores were in contact with distilled water or oil on 1% water agar in darkness at 18°C.

er RH (Table I). Spores stored en masse respond to atmostpheric humidity and lose germination at slower rates than dispersed spores.

3. *Semipermeability Changes*

Sudden wetting of dried spores with water results in enhanced leakage of cell electrolytes into the ambient water, and the amount of leakage is greater for spores stored at 0% RH than at higher humidities (Table II). Again, spores stored at 0% RH have lower germination rates when placed on water than when germinated in oil. These observations suggest that the sudden rehydration of dried urediospores irreversibly disrupts the semipermeability of the plasmalemma and thereby prevents germination. Spores stored at high humidity (90% RH) had low germination rates on both water and in oil and no increase in electrolyte loss (Table II), indicating that loss of membrane semipermeability was not the cause of reduced ability to germinate.

4. *Respiratory Changes*

Patterns of respiration indicate how spore activity varies when spores are stored at different relative humidities (RH; Fig. 1). Respiratory activity is high for spores stored in 90% RH, but germinability at this RH declines rapidly after 10 days storage. The loss of germinability apparently is due to the loss of energy reserves, because 34% of the spores failing to germinate on water agar after 22 days storage germinated on glucose agar. About one-third of the respiration of spores

Table II

Effect of Relative Humidity during Storage on Leakage of Electrolytes from Urediospores of *Puccinia graminis* f. sp. *tritici*[a,b]

Relative humidity (%)	Storage for 7 days			Storage for 14 days		
	Resistance (1000 Ω)	Germination (%)		Resistance (1000 Ω)	Germination (%)	
		In water	In oil		In water	In oil
0	70.7	39	89	73.8	10	85
20	100.5	61	92	105.0	72	81
50	98.6	71	89	82.0	28	78
90	101.0	54	87	97.0	0	16

[a]J. B. Rowell, unpublished data.

[b]Electrolyte leakage determined by the resistance of the filtrate after 1 hr extraction of 50 mg of urediospores in 100 ml double glass-distilled water. Duplicate 50-mg spore lots were sampled to determine germination in direct contact with water on polyethylene film and in oil on 1% agar.

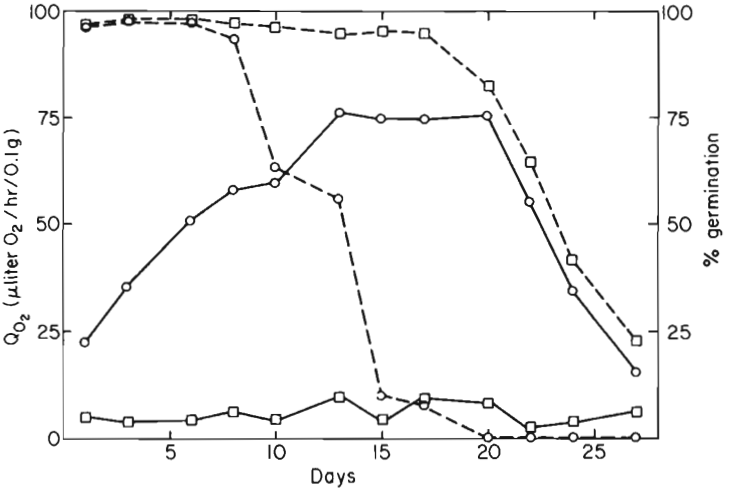

Fig. 1. Effect of relative humidity during storage on respiration and germinability of masses of dry urediospores of *Puccinia graminis* f. sp. *tritici* (J. B. Rowell, unpublished data). (○——○), Respiration at 90% RH; (○- -○), germination in oil on water agar at 90% RH; (□——□), respiration at 80% RH; (□- -□), germination in oil on water agar at 80% RH.

stored 13 days at 90% RH was judged from experiments with heat-killed spores to be due to contaminating microorganisms, which are evident as traces of fungal mycelia after 20 days storage. Thus storage in 90% humidity supports active metabolism in the spores, which increases moisture content by the accumulation of metabolic water and reduces longevity by exhausting metabolic reserves. Spores stored at 80% RH (Fig. 1) have a steady, low respiratory rate for 27 days. Spore germination at this humidity started to decline after 17 days of storage and was 23% at 27 days. Germination of these spores is not increased on 2% glucose, which suggests that the nature of the loss in germinability at 80% RH is not simply from depletion of nutrients. Spores stored at 50% RH have had no detectable levels of respiration, and more than 90% of the spores have germinated after 27 days.

5. *Poststorage Effects*

Dry urediospores of *P. graminis* have a wide temperature tolerance. These spores reportedly survived exposure for up to 10 hr at 60°C (Hwang, 1942). Subfreezing temperatures were once considered lethal to urediospores. The discovery (Loegering *et al.*, 1961) that dry urediospores survive cryogenic freezing at −196°C when the retrieved spores are thawed for a few minutes at 37°C indicated that ultracold tempera-

tures were not lethal but induced a heat-reversible dormancy. Subsequent studies (Bromfield, 1964) revealed that germinability was restored in spores frozen at a range of temperatures from −1.1° to −196°C by exposures to either a "heat shock" at 40°C for 5 min or to vapor-phase hydration for 16 to 24 hr in a sealed container over water at 20°C. Subsequently, Maheshwari and Sussman (1971) showed that frozen spores thawed at 20°C were supersensitive to liquid water, which caused an irreversible injury that prevented spore germination. This hydration injury to the spore increased leakage of metabolites, suppressed incorporation of isotopic carbon, and rapidly diminished respiratory activity.

Clearly, both dehydration and freezing condition urediospores to injury by sudden wetting with water. The report by Grieve and Povey in 1981 that differential freezing reverses osmotic flow across a membrane suggests that frozen urediospores are dehydrated osmotically to a state comparable to that of desiccated spores. The mechanism by which sudden wetting with water affects the plasmalemma to disrupt cell permeability remains unclear.

It is evident that water relations during storage profoundly affect urediospore germinability. Excessive drying irreversibly injures spores, and excessive moisture vapor fosters respiration that exhausts metabolite reserves or other essential components required for germination. Spore germinability is also reduced by abrupt changes in spore moisture when spores are retrieved from storage.

B. METHODS OF STORAGE

No special procedures are necessary for urediospores kept for short periods of time. Storage in a covered container at room temperature and humidity are satisfactory when freshly harvested spores will be used within a few days. Under these conditions, however, germinability will decline within a week or so. Freshly harvested spores stored in a sealed container at 4°C may be germinable from several months to a year. Under refrigeration, urediospores of *Puccinia coronata* and *P. recondita* appear to survive better than those of *P. graminis*. At the Cereal Rust Laboratory, freshly collected urediospores from the leaf and stem rusts of wheat and oats are stored temporarily in open tubes at 4°C over a solution of 82.4 g KOH/100 ml water with a vapor pressure that gives about 20% RH. This storage is satisfactory for routine inoculations within a few weeks after collection without any conditioning treatment of the spores (see Section IV,A). Storage of urediospores for extended periods of time in inoculation oil is un-

satisfactory at either room or refrigeration temperatures, because the spores settle to the bottom of the container and provide a source of sufficient nutrient and moisture to support the growth of saprophytic microorganisms.

Urediospores can be preserved for extended periods of time either by drying and storing the spores in a vacuum or by freezing and storing the spores at very low temperatures. The vacuum-drying method is simpler and less expensive but is less dependable than storage in liquid nitrogen cryostats for periods of 5 or more years.

The vacuum-drying method (Sharp and Smith, 1957) was developed as a modification of the lyophilization technique used to preserve bacteria and some fungi. The vacuum-drying method differs from lyophilization procedures in that the spores are not frozen in a hygroscopic embedding medium but are dried en masse and sealed in tubes under vacuum. Spores preserved by this method are not inert (Wynn *et al.*, 1966), and preservation results from the suppression of biochemical changes by the reduced availability of water and oxygen.

A vacuum-drying system consists of a manifold to hold lyophil tubes connected to a moisture trap and evacuated by an oil vacuum pump capable of lowering pressure to about 0.1 mm Hg. Either a cold trap chilled by dry CO_2 in acetone, or a chemical desiccant trap containing anhydrous $CaSO_4$ or silica gel is suitable for a moisture trap. For use, the opened tubes are placed in a sealed container over water for 16 to 24 hr to allow gradual rehydration of spores and prevent injury on direct contact with water. Loss in germination can occur during the drying of the spores, and germination gradually declines with time in storage. Vacuum-dried spores survive better in tubes stored at 4°C than at room temperatures. Survivability can vary for spores of a given rust culture that are produced at different times in a glasshouse. The major difficulty of this method is determining when spores have reached the optimal moisture content for maximum preservation. Too much desiccation is lethal, and insufficient desiccation shortens storage life of urediospores. Generally, spores dried for 2 to 3 hr in a vacuum of 10 to 250 μm Hg with a moisture trap will be sufficiently dry (about 2% moisture) for extended storage. Small quantities (10–50 mg) of spores, however, will dry faster than large quantities (500 mg).

The most reliable method for long-term preservation of urediospores is to store them in sealed tubes in a liquid nitrogen (LN) cryostat (Loegering *et al.*, 1961). Cultures stored at the Cereal Rust Laboratory during 1963 in a LN cryostat gave high rates of germination and infection in 1981. A readily available source of LN is needed, because the cryostats require refilling with LN at regular intervals to maintain the temperature in the range of −160 to −193°C. Dry spores are placed in

small tubes made from 5- to 7-mm-diameter borosilicate tubing; the tubes are then sealed with a high-temperature gas torch and are placed in the LN cryostat for freezing and storage. Heat-resistant borosilicate glass tubes are required to withstand the temperature stress of freezing and thawing. Several sealed tubes are mounted on a "cane" placed in the cryostat. Tubes must be sealed completely to prevent entry of LN, which could cause an explosion during thawing. Leaks in sealed tubes can be detected by external gas bubbles that appear when the tube is immersed in a 40°–45°C water bath or by penetration of a dye solution into tubes when they are immersed in the dye at 4°C. At the Cereal Rust Laboratory, canes removed from LN are immediately inserted into a 5-cm cast iron pipe to contain any explosive shattering of the tubes due to LN leakage. Occasionally, tubes have exploded on opening the tubes several days after removal from LN, presumably because of gaseous nitrogen trapped under pressure in the tube. Thus operators should use protective eye and face shields as well as gloves whenever handling retrieved tubes.

Because frozen spores thawed at room temperature germinate poorly when directly wetted with water, a conditioning treatment on retrieval from LN is used to restore good germination and infectivity. Exposing the retrieved spores to heat by immersing the sealed vial in a water bath at 45°C for several minutes or rehydrating the spores at 80–90% relative humidity for 16 to 24 hr restores germinability and infectivity. Once spores are frozen rapidly by immersion in LN, the vials do not have to be immersed continuously in LN, because the low temperature of the gaseous nitrogen in the cryostat preserves spores. Furthermore, spores in sealed vials remain viable after repeated cycles of freezing and thawing. Because rapid frosting of canister and tube surfaces on removal from the cryostat obliterates labels, a system for storing culture tubes by location in the cryostat ensures retrieval of a given culture (Leath *et al.*, 1966). (A small paper label inside each tube identifies the culture.)

The disadvantages of LN storage are the expense and the hazardous properties of LN. The N_2 emitted continuously from the cryostats can displace oxygen in unventilated, confined spaces and become an asphyxiation hazard. Furthermore, LN is extremely cold, and it, as well as canisters, canes, and tubes removed from the cryostat, can instantaneously cause severe freezing injury on contact with skin. Operators of LN refrigerators should be instructed in safe handling techniques and be provided with approved protective equipment.

Unpublished studies by M. Prescott (personal communication) demonstrated that urediospores stored at −55°C in ultralow-temperature freezers and heat treated at 45°C for several minutes after retrieval

from storage gave nearly as good survival as spores in LN. Bulk storage of urediospores in such freezers is convenient for accumulating large amounts of inoculum. I have successfully stored 0.5 g of spores sealed in 10- by 15-cm flat polyethylene bags at −45°C for 2 years with negligible loss of germinability or infectivity. In my method, the freshly collected spores are held at 50% RH for 2 days (to dry the spores partially) and then placed in the polyethylene bag, which is heat sealed about 3 cm below the opening. A dated label identifying the culture is inserted in the remaining pocket and held in place by a second heat seal. Initially, the bags are placed flat on the bottom of the freezer for several hours to freeze the spore mass rapidly, and the bags are subsequently bundled together for storage. On retrieval the bag is immersed in a water bath at 45°C for 5 min to restore germinability. Commercially available plastic tubes for cryogenic storage are also satisfactory for bulk storage of spores.

A variation of the ultralow-refrigeration method was developed to allow quick storage of rust isolates from annual race surveys for future studies. Several infected leaves with numerous sporulating uredia of an isolate are placed in a 5- by 15-cm labeled glassine bag. The bags are placed in a vacuum desiccator containing anhydrous $CaSO_4$, and the air is evacuated to 12 to 15 mm Hg. After several hours, depending on the number of samples in the desiccator, the leaves become visibly dry and shriveled. Then the bags are removed from the desiccator and immediately placed flat on the bottom of the freezer. Several hours later the refrigerator is opened, and the bags are quickly transferred to a storage box inside the refrigerator. When a collection is retrieved for use, the glassine bag is inserted into a polyethylene bag for protection against wetting the sample, and the bags are suspended in a water bath at 45°C for 10 min. After this heat treatment, the glassine bag is placed in a sealed chamber over 23.5% KOH (giving 80% RH) at room temperature for at least 4 hr of hydration before the spores are used as inoculum. Germination percentage of spores stored by this method is less than that of samples collected from the same source material and stored under LN; however, more than 50% of the spores germinate after 1 year of storage, which is adequate for routine inoculations.

IV. Preparation of Inoculum

A. CONDITIONING TREATMENTS

Freshly harvested urediospores do not require conditioning before use as inoculum, and neither do spores held for several days at room

temperature and relative humidities between 50 and 80%. In my experience with controlled inoculations, freshly harvested spores were more dependable than stored spores for reproducible control of the infection per unit of spores from experiment to experiment.

Spores stored by either vacuum drying or freezing require heat and/or hydration treatments to obtain maximum effectiveness as inoculum. The advantages of heat treatment of stored urediospores are the brevity of the treatment period and the broad range of effective temperatures. The effective range is 36° to 60°C, and treatments at 40°C have been equally effective for exposure times of 0.25 to 40 min. Apparently, the critical aspect for heat treatment is to make sure that the entire spore mass is heated to the effective temperature. Furthermore, the spores can be thawed at room temperature and subsequently reactivated by a heat treatment (Loegering and Harmon, 1962; Bromfield, 1964), after which retrieved spores have about the same longevity as fresh spores (Bromfield, 1964).

Reactivation of vacuum-dried or frozen spores by vapor-phase hydration is as effective as heat treatment, but it takes longer (Bromfield, 1964). Hydration over pure water in a closed container at room temperatures for 16 to 24 hr is generally effective. The duration of an effective hydration treatment is determined by the time required for moisture equilibration, which is governed by the surface area and volume of the spore mass, the volume of air in the container, and the exposed surface area of water. I prefer to use a 23.5% KOH as the source of water vapor, which gives 80% RH at 20°C, to avoid moisture condensation from chance drops in temperature.

B. MEASURING INOCULA

A convenient measure of inocula is by weight. The number of urediospores per unit weight, however, can vary with its moisture content or by presence of contaminants. Spore number per unit weight often is determined by using a hemacytometer. I dilute spores in a 1:1 mixture of light inoculating oil and light petrolatum, and I use a 5-mm platinum loop to place a uniform drop on the grid surface. An average number of 450,000 spores/mg was found in repeated trials with freshly harvested spores from infected plants grown in the glasshouse under good light and temperature conditions. During the poor light conditions of winter, however, the average number of freshly harvested spores was 620,000 per mg.

The number of urediospores in inoculum preparations can be determined with a nephelometer if a mixture of 2 ml of inoculation oil with

5 ml of carbon tetrachloride is used to suspend the spores. This mixture has a density of 1.35 that will hold spores in suspension adequately for a nephelometric determination (the density of urediospores is approximately 1.35). The procedure is as follows: pipet 2 ml of urediospores suspended in light inoculation oil into a nephelos cuvet; add 5 ml of CCl_4 and mix it thoroughly; determine the number of nephelos units in a Coleman nephelometer in comparison to Coleman nephelos standard 21 expanded by a factor of 3; subtract the number of nephelos units for a sporeless blank of the oil–CCl_4 mixture; and multiply the remainder by 3794 to obtain the number of spores per milliliter in the initial inoculum suspension in oil.

C. CARRIERS

Most inoculations with urediospores require dilution of the inoculum in a carrier for efficient, uniform dispersal of the spores. Dry spores are generally mixed in a ratio of 1:4 with dry carriers such as talc, diatomaceous earth, or Lycopodium powder for dusting applications. Large-scale inoculations in the field with the dry carriers can be applied with commercially available hand-operated or mechanized backpack dusters. Small-scale inoculations in the glasshouse often use a simple "puff" duster constructed with a large test tube, a two-hole stopper in which two pieces of glass tubing bent at right angles are inserted, and a rubber atomizing bulb attached to one tube. Dust inoculation is inefficient for depositing spores on target plants, is subject to large losses from drift, and contaminates the work area with large numbers of airborne spores.

Water serves as a carrier of urediospores for hypodermic inoculations and occasionally for spray applications to foliage. Spores conditioned initially by vapor-phase hydration should be used to avoid the injury induced by suddenly wetting dry spores with water. Because the hydrophobic urediospore surface resists wetting and dispersal in water, surfactants are often used to prepare spore suspensions. However, I have not found a satisfactory surfactant for use with urediospores. I have tested several, including Tween 20 and Aerosol OT, which have been used by some workers to suspend spores in water. At 100 ppm, they failed to disperse 20 mg of urediospores completely in 5 ml and reduced the percentage of spore germination as well as the length of germ tubes. At 10 ppm, germination was normal in many surfactants, but spore wetting and dispersal were negligible.

Urediospores can be suspended satisfactorily in water by first preparing a paste of spores. A spore mass is placed in a small beaker; one drop

of water is then added and kneaded into the spore mass with the rounded end of a glass rod. This process is repeated by adding one drop of water at a time until the moistened spore mass has the pasty consistency of a heavy cream. At this point the bottom of the beaker is placed in the bath of an ultrasonic cleaner for about 1 min, and the remaining volume of water required for the final suspension is added during the sonication. This step degasses the spore surfaces, which improves spore wettability and yields almost complete dispersal of the spores in water. Good but incomplete suspensions leaving a film of unwetted spores on the water surface are obtained if the sonication is omitted. Spores in suspensions prepared by this method germinate normally.

Urediospores are highly lipophilic and are readily wetted and suspended by oils and other nonpolar fluids. Some highly refined, nonphytotoxic petroleum oils make excellent carriers for rust inoculum (Rowell and Hayden, 1956; Rowell and Olien, 1957). These oils are commercial products synthesized catalytically from selected petroleum fractions. They are mixtures of various saturated hydrocarbon compounds in proportions that will vary with the source of the crude petroleum. The oils I have found to be most effective have physical characteristics similar to Isopar M (Exxon Co., United States) as follows: ASTM initial boiling point 207°C, final boiling point 260°C, specific gravity 0.78, and flash point 77°C. Two types of oils, isoparaffinic and naphthenic, have been satisfactory carriers. Isoparaffinic oils such as Isopar, Mobilsol 100 (Mobil Oil Co.,), and Soltrol 170 (Phillips Petroleum Co.) are alkane hydrocarbons, which have a saturated linear chain or branched structure. A naphthenic oil such as Odorless Insecticide Base Oil W-4 (American Mineral Spirits Co.) consists of cycloalkane hydrocarbons, which have saturated ring structures. These oils have not differed significantly from each other in comparative controlled inoculations in the number of spores deposited on foliage, the percentages of germination and host penetration, and the numbers of infections. All of these oils have extremely low phytotoxicity and are used also as diluents for insecticides. These oils are most successful as carriers when spray deposits are light and invisible. They are moderately volatile, however, and visible deposits should be allowed to evaporate before exposing inoculated plants to dew. These are volatile hydrocarbon solvents classed as combustible liquids and should be kept away from heat, sparks, and open flame. Operators also should avoid prolonged skin contact or breathing oil vapors.

In addition to industrial oils, I have tested several purified hydrocarbon compounds as carriers of urediospores (Table III). In general, the

Table III

Effect of Some Hydrocarbon Fluids as Inoculation Carriers of Urediospores of *Puccinia graminis* f. sp. *tritici*[a,b]

Hydrocarbon compound	Empirical formula	Boiling point (°C at 760 mm Hg)	Infections per leaf
Alkanes			
n-Heptane	C_7H_{16}	98.4	4.7
Trimethylpentane	C_8H_{18}	99.3	5.2
Octane	C_8H_{18}	125.6	5.7
Nonane	C_9H_{20}	150.7	10.0
n-Decane	$C_{10}H_{22}$	174.0	13.0
Dodecane	$C_{12}H_{26}$	214.5	18.7
Soltrol 170	—	196–261	25.9
Cycloalkanes (naphthenes)			
Cyclohexane	C_6H_{12}	80.7	3.7
Cycloheptane	C_7H_{14}	118.1	4.0
Cyclooctane	C_8H_{16}	148.0	7.9
Odorless Insecticide Basc Oil W-4	—	196–246	16.0

[a] J. B. Rowell, unpublished data.

[b] Infection data from Little Club wheat seedlings inoculated by atomization of 0.2 ml of a suspension containing 0.5 mg of spores/ml.

higher the boiling point of the compound, the greater the amount of infection. Germination of urediospores from each suspension dispersed in an oil drop on water agar, however, did not differ significantly among the compounds, with the exception of cycloheptane, in which spore germination and germ tube length were reduced appreciably. In other tests, plants were inoculated with spores carried in some of these compounds at two concentrations and examined for pathogen development (Table IV). The major difference observed between the carrier oils was in number of spores deposited on the leaves. The higher the boiling point of the carrier oil, the greater the number of spores deposited per square centimeter of leaf surface, which mostly accounts for the greater number of infections observed.

A variety of inert, synthetic fluids are effective carriers of urediospores. Trichlorotrifluoroethane (Freon 113, E. I. Dupont De Nemours & Co.), boiling point 47.6°C, is an effective carrier (Miller, 1965). Spores float in this dense fluid (1.57 g/cm^3), and spore germination declines after several hours' exposure. This fluid has a low viscosity (0.66 centipoise) and boiling point, which results in rapid flow through spray nozzles. In comparative tests as carriers of urediospores, tri-

Table IV

Effect on Spore Deposition, Germination, Appressorium Formation, and Infection of Selected Hydrocarbon Fluids as Carriers of Urediospores of *Puccinia graminis* f. sp. *tritici*[a,b]

Hydrocarbon compound	Spores/cm²[c] No.	Spores/cm²[c] % of control	Germination[c] (%)	Appressoria/cm²[c] No.	Appressoria/cm²[c] % of control	Infection per leaf[d] No.	Infection per leaf[d] % of control
Heptane	122	18	77.3	38	16	4.5	17
Nonane	168	25	87.6	51	21	12.3	47
n-Decane	467	70	73.8	132	54	13.3	51
Dodecane	667	101	66.9	203	83	19.2	74
Control (Soltrol 170)	663	—	81.2	245	—	26.0	—

[a]J. B. Rowell, unpublished data.
[b]Data from Little Club wheat seedlings inoculated by atomization of 0.2 ml of a urediospore suspension.
[c]On leaves inoculated with 20 mg urediospores/ml.
[d]On leaves inoculated with 0.5 mg urediospores/ml.

chlorotrifluoroethane deposited fewer spores, had more spore aggregates, and gave fewer infections than light mineral oils. Percentages of spore germination and appressorial formation were similar in both carriers.

Several perfluorochemicals, (FC-43 and FC-77, Fluorinert electronic liquids, 3M Co.), although more costly than mineral oils, are also effective carriers (Bushnell and Rowell, 1967). These fluids have remarkably low toxicity and a high capacity to dissolve oxygen and carbon dioxide. Composed mostly of perfluorotributylamine, FC-43 has excellent spray characteristics (viscosity 2.8 centipoise at 25°C), depositing spores at rates comparable to light mineral oils, but more spore aggregates are present. The liquid deposited on plant surfaces evaporates slowly (vapor pressure 0.3 mm Hg at 25°C; boiling point 170°C). Spores applied in sprays with this carrier germinated, formed appressoria, and infected plants at frequencies similar to that for light mineral oils. Dispersed spores rise to the surface of this dense fluid (1.88 g/cm³ at 25°C). FC-77, a mixture of perfluorinated 8-carbon compounds is too volatile (vapor pressure 30 mm Hg at 25°C; boiling point 99°–107°C) for usage in controlled sprays but is useful for topical applications of suspended urediospores directly to leaf surfaces with a cotton swab. These fluids should be effective carriers for hypodermic inoculations.

D. GERMINATION TESTS

Ideally, a test for estimating the inoculum potential of a urediospore sample should evaluate the capacity for completion of the infection process, which includes germ tube initiation, germ tube growth, and germ tube differentiation of infection structures. Generally, investigators only test the percentage of spores that produce germ tubes, and even those tests are subject to errors dependent on the method employed. For instance, the percentage of germination obtained on water agar at 21°C for a sample of urediospores of *P. graminis* f. sp. *tritici* was 27% for spores smeared across the agar surface, 73% for spores dusted onto the surface, and 99% for spores dispersed in a drop of oil and placed on the surface. These different results are due to differing rates of spore hydration, and the extent of the variation between methods is affected by the moisture content and age of the spores (see Section III,A). In addition, the outcome of a germination test is affected by temperature, light, substrate surface, water source, and air purity.

1. *Temperature and Light*

Temperature is not a major limiting factor for the initiation of germination because of the relatively wide range of optimum tempertures, 15°–23°C (Burrage, 1970). Light temporarily inhibits the *in vitro* initiation of germination of spores hydrated after storage (Givan and Bromfield, 1964), but appears to have little effect on freshly harvested spores (Burrage, 1970).

2. *The Substrate Surface*

The nature of the substrate surface is crucial to the successful determination of germination rates, because the surface can alter the distribution and orientation of spores, the way germ tubes are initiated, and the subsequent pattern of germ tube growth. Ideally, the substrate surface should induce behavior similar to that on the host surface.

Urediospores landing on the host generally are oriented with the flattened long axis of the spore contiguous to the hydrophobic cuticle. Swellings appear at all pores when dew is deposited, and the germ tube initiates from a pore swelling in contact with the cuticle. The tube grows closely appressed to the cuticle and becomes oriented at right angles to the long axis of the host surface. Aborted short branches may form at right angles to the tube when the tip encounters surface discontinuities such as the wall boundaries between epidermal cells, sclerenchyma ridges, or stomata. Appressoria generally form when the

germ tube encounters the slight depression between closed stomatal guard cells. Lewis and Day (1972) have shown that the cuticular crystal lattice governed germ tube development (see Staples and Macko, Chapter 9, this volume).

Urediospores landing on aqueous surfaces behave atypically as a result of the hydrophobic surfaces of the spore wall. On water, the spores quickly gather into a mass at the bottom of the meniscus. On water agar the spore often makes minimal contact with the surface, and the germ tube develops from a pore opposite to the point of contact. The tube elongates into the air until the germ tube mass rolls the spore over and the tube falls to the substrate surface. Subsequent germ tube growth may be above, on, or below the substrate. Occasionally, the germ tube protoplast will extrude from the tip of a germ tube, and the germling dies. Extrusion is induced in 60 to 80% of the germ tubes grown on 1% Noble's agar (Difco Co.) in distilled water by initiating spore germination in the dark at 18°C for 3 hr followed by transferring the germlings to 30°C. This temperature regimen is similar to that used to induce the formation of infection structures (Maheshwari *et al.*, 1967).

Urediospores deposited on hydrophobic plastics orient flat against the surface. When dew is deposited on smooth polystyrene (Burrage, 1969), the germ tube initiates from a pore adjacent to and grows appressed to the surface. These tubes branch dichotomously at regular intervals, but only one branch continues to grow and the other aborts. These germ tubes do not form appressoria. Spores in oil drops on water agar behave similarly. Germ tube growth, development, and appressorium formation on polyethylene film etched with many parallel scratches by fine aluminum oxide sandpaper resembles that on host surfaces (Rowell, 1967), and this method may be a useful test of the potential infectivity of inoculum.

3. *Water*

Water quality is a major source of error in tests of urediospore germinability. Urediospore germination is inhibited by a wide variety of organic and inorganic substances that contaminate water. For many years the tap water in my laboratory came from deep wells and had 200 ppm hardness. Spores germinated poorly, if at all, in this water. A single distillation of this water in a metal still improved the water quality for urediospore germination but did not support as good germination as water from a second glass distillation. Distillation does not necessarily assure water of sufficient purity, because inhibitory

inorganic and organic substances can be carried over to the distillate in tiny droplets formed from bursting bubbles during vigorous boiling.

Substances toxic to urediospore germination also may be released or dissolved from the walls of storage or experimental containers. Urediospore germination is inhibited in soft glass containers cleaned by dichromate–sulfuric acid solution followed by five rinses in distilled water. This toxicity can be eliminated by soaking the glassware in 0.05 *M* phosphate buffer in distilled water overnight and rinsing five times in double-distilled water. I have found that various commercial humidifiers introduce inhibitory substances, especially those made of copper or brass, when used as a source of finely atomized mists for urediospore germination and infection. Tests of standing water from a humidifer's copper reservoir by my assay (see chapter by Rowell, Vol. II) indicated that toxicity to germinating urediospores equaled 19.9 ppm Cu^{2+}.

Water purified by deionization in mixed-bed, ion-exchange columns used intermittently is toxic to urediospore germination, apparently because of microbial growth on the resins. Similar microbial growth and toxicity may occur in plastic piping used for distilled water distribution systems.

4. An Assay Method

In my experience, the most reliable method to test the rate of urediospore germination has been to disperse spores in a drop of oil on water agar. The details of the procedure are as follows: Urediospores other than freshly harvested spores are conditioned by hydration in a sealed container at either 50 or 80% RH for at least a day before the test. The test substrate consists of 1% Noble's agar (Difco Co.) in double glass-distilled water. The agar is poured into sterile Petri dishes 24 hr before the test to permit drying of the surface water film and to prevent spreading of the oil drop. A 1:1 mixture of light inoculation oil such as Isopar M and light petrolatum U.S.P. is used as the carrier for the spores. Spores are handled in an atmosphere $\geq$50% RH to prevent artifacts caused by rehydration injury. A 5-mm-diameter platinum loop is used to place individual drops of the germination oil on the agar surface. A similar drop of germination oil is placed on the surface of a clean glass slide, and an extremely small mass of spores, about one half to one-eighth the size of a pinhead, is dispersed in this drop. One drop of the suspended spores is transferred with a 3-mm-diameter platinum loop to each oil drop on the agar. It is important to touch the platinum loop lightly to the top surface of the oil drop and not smear the oil drop

across the agar surface. The inoculated plates are incubated in the dark at 18°C for 16 hr, and the number of germinated spores per 100 in each drop are counted. Although the percentage of germ tube initiation can be determined after 4 hr of incubation, the longer incubation time permits and evaluation of the vigor and normalcy of germ tube growth and development.

The germ tubes from freshly harvested urediospores in an oil drop on water agar grow in a sympodial pattern similar to that observed on plastic surfaces (Burrage, 1969). Occasionally, a germ tube will penetrate and grow into the agar substratum, but I have never observed a germ tube that penetrated the oil–air interface. Components of the light isoparaffinic oils used as inoculum carriers gradually oxidize during prolonged storage, and in such oils germ tubes grow atypically straight and unbranched with a tapered tip. Such oils should not be used for quantitative studies but are satisfactory for routine inoculations.

V. Preparation of Host

Plants of uniform size and development are required for experiments concerned with rust infection under artificial conditions. Temperature, light, and moisture before and after inoculation affect both plant development and stem rust infection.

A. PREDISPOSITIONING FACTORS

A satisfactory standard procedure for growing uniform wheat seedlings for controlled inoculation is as follows: Two seeds are sown in a mixture of soil, sand, and peat at each point of a pentagon around the inner edge of a 6.5-cm-diameter clay pot and covered with 1 cm of soil mixture. After watering, the pots are placed on a bed of sand heated to 25°C in the glasshouse. When the first coleoptiles become visible, the pots are transferred to watering trays in a growth chamber programmed for 18° ± 1.5°C and a 14-hr photoperiod from cool white fluorescent lamps that deliver 16,000 lux of light at the tips of fully emerged first foliar leaves. Seven days after planting, the seedlings have erect first foliar leaves about 10 cm long with the ligule present at the base of the blade. The five most uniform leaves in each pot are then selected for inoculation, and the remainder are removed.

Germination of wheat seed initiated in warm soil is more rapid and uniform than in cool soil. Continuous exposure to either high or low soil temperatures, however, results in either long, narrow, recumbent or short, broad, upright leaves, respectively. The transfer of plants to the cool environment when the tips of the coleoptiles emerge from the soil gives rapid development of upright, uniform, moderate-sized leaves suitable for inoculation.

The intensity, quality, and duration of light during the growth of seedlings prior to inoculation affects the amount of infection produced on a susceptible host by a unit of inoculum. During the short, cloudy days of winter in Minnesota, plants grown in the glasshouse under natural light prior to inoculation will have only about 10% of the infection that is produced by the same inoculum on plants grown with natural light supplemented by 8 to 12 hr of 10,000 lux from fluorescent lights as described in Section II,B. Sharp *et al.* (1958) also noted variation in infection success in seedlings exposed to different preinoculation light intensities. Daly (1964) compared the effect of light from fluorescent lamps alone to the light from fluorescent plus incandescent lamps in pre- and postinoculation environments, using a 13-hr photoperiod. His data suggest that preinoculation plant growth under fluorescent plus incandescent light was more favorable for infection than fluorescent light alone, but that the incandescent component was deleterious for infection after inoculation.

Soil moisture also influences infection. Waterlogged soil inhibits seedling growth and favors root and seedling blights, and drought stress prior to inoculation also renders the growing plants less receptive to infection. I have tested short periods of drought stress imposed at various stages in the growth of test plants prior to inoculation by withholding water from replicated pots until incipient wilt symptoms were evident, at which time the pots were watered and the plants recovered. Drought stress at any time during the 3 days prior to inoculation markedly reduced infection. Burrage (1970) found that drought stress imposed immediately before inoculation diminishes infection success with increases in the time that water was withheld.

B. HOST CONFIGURATION

The form and growth habit of the plant organs are also variables that affect rust inoculations. The first foliar leaf of the wheat seedling initially grows upright and generally has a right-hand twist. When seeds

are planted vertically with the embryo down and the groove facing the center of the pot, the seedlings emerge with the first foliar leaf facing the pot rim. The junction between blade and sheath appears about a week after planting, and the blade ultimately folds to a recumbent position at this junction. The length:width ratio of the lamina of the first foliar leaf varies with environmental conditions and differs among wheats. Winter bread wheats and durums often have longer and narrower blades than spring bread wheats. Stomata are present on both leaf surfaces but generally are more numerous on the adaxial surface, and the number per square centimeter varies slightly with growing conditions. The longitudinal curling of wheat leaf blades under drought stress is controlled by rows of bulliform cells on the adaxial surface parallel to the long axis of the blade. The transverse topography of the adaxial leaf surface is more uneven than the abaxial surface, which hinders direct microscopic observations on spore germination and appressorial development.

The numerous tall, limber tillers of adult wheat plants are difficult to inoculate uniformly. The tillering stage commences after the third foliar leaf has emerged about 3 to 4 weeks after planting. The ultimate number of tillers produced is dependent on plant genotype and environmental conditions. Winter wheats require vernalization (exposure to temperatures below 7°C for about 6 weeks) to make the transition from the tillering to the shooting stage. A 1-hr interruption of the dark period with incandescent light during the tillering stage stimulates the formation of floral initials and shifts development from the tillering to the shooting stage. Careful control of light regimens often will limit development to two or three tillers. The tillers mature at slightly different rates.

The adult plant generally has six internodes, the first two of which are short and usually below ground. The fully developed tiller usually has four large foliar leaves at the heading stage. Plants are often inoculated at this stage, when most of the stem surface including the peduncle is covered by the leaf sheaths, and the plants, about two-thirds of their final height, are comparatively sturdy. As the peduncle elongates and the head weight increases from the developing grain, the tillers become top-heavy and readily break if unsupported. Wheat cultivars vary widely in rate of development, and infection frequencies per unit of inoculum may vary with growth stage (Rowell and McVey, 1979). Hence, comparative studies of infection frequencies on diverse cultivars under artificial conditions require different planting dates to synchronize the stage of host development at inoculation.

VI. Procedure of Inoculation

Innumerable methods have been devised to inoculate cereal plants with urediospores of the cereal rust pathogens (Browder, 1971). Carriers for inoculations are discussed in Section IV,C. I limit my discussion here to procedures used for quantitative control of deposited inoculum.

A. DUSTING

A variety of settling towers have been developed for quantitative deposition of dry urediospores on cereal leaves. This method requires the reproducible generation of a uniform dust cloud of spores in the chamber. The settling tower of Eyal *et al.* (1968) is representative of this inoculation method. The tower consists of an upper cloud chamber separated by a shutter from a lower settling chamber where the exposed leaf blades are taped to the surface of a disk. A quantity of spores are explosively ejected into the upper chamber from tubing connected to a modified (Crosman) CO_2 pistol (Lange *et al.*, 1958). The shutter is used to regulate the deposition period and to exclude spore clumps that settle from the cloud more rapidly than individual spores. The chamber should be grounded, because electrostatic charges may induce irregularities in the spore cloud and spore deposition. A major disadvantage of settling towers for inoculation is the considerable potential for contaminating the surrounding work area and the difficulty of cleaning all components of adhering spores between successive inoculations. The exposure of only one surface of a single leaf per plant in this settling chamber may be a disadvantage for epidemiology studies.

Melching (1967) devised a turntable to inoculate adult cereals with rust pathogens in a large settling tower. The plant pots are mounted on a series of small plates that are arranged symmetrically near the periphery of a large turntable. As the turntable makes one revolution in one direction, the plates rotate the plants once in the opposite direction. A spore cloud is ejected by modified CO_2 pistol into the large cylindrical tower, and spores are deposited by impinging rather than settling onto the host surfaces. Theoretically, rotation of the plant as it moves through the spore cloud exposes all host surfaces to inoculum. My observations on adult plants inoculated by this method, however, have indicated that spores are deposited heavily on one side of the leaf blade and sparsely on the opposite side. This inequity in spore deposi-

tion apparently results from the asymmetrical orientation as leaf blades flap about as a result of effects of centripetal force and air resistance on the rotating plant during its circular path around the chamber. Furthermore, more spores are deposited on the upper than the lower plant parts presumably because of the creation of a gradient in the descending spore cloud as the plant tops sweep through it. In modifications of this procedure, a mist is generated in the chamber by atomizing spores carried in oil (Politowski and Browning, 1975) or an inert perfluorinated fluid (Mortensen *et al.*, 1979).

B. SPRAYING

Spraying is a convenient means of inoculating cereal plants with urediospores. However, pressure spray nozzles are unsatisfactory for inoculation because of the wide range in droplet size and the tendency of the small orifice to clog. Venturi atomizers, in which the spore suspension is broken into fine droplets by the rapid passage of air across the end of the fluid delivery tube, are generally used. This type of sprayer produces a solid cone of spray droplets with a pattern of spray deposition that is most dense in the center and diminishes progressively toward the periphery. Furthermore, the velocity of the spore droplets and spores decelerates rapidly with distance from the sprayer, so that they soon have insufficient inertia to penetrate the boundary layer and deposit on plant surfaces. I have used a spray chamber with a moving column of air to increase the uniformity of droplet velocity and pattern (Rowell and Olien, 1957). Air drawn around the atomizer carries the spray across plants rotating on a turntable, passes through the rear opening, and is exhausted outside of the work area. The effective spray pattern at 38 cm from the atomizer is 16 cm in diameter. This method was rapid and useful for inoculating seedling plants, but the small cross-sectional area of effective spore deposition limits the utility for inoculating adult plants.

Commercially available mechanical backpack mist blowers are readily adapted to inoculate field plants uniformly (Rowell and McVey, 1979). A small orifice is required for the fluid delivery nozzle to adapt the sprayer for applying low volumes of oil. Application rates equivalent to about 6 liters/ha can be achieved. Frequent agitation is needed to maintain a uniform suspension of spores; therefore, a plastic bottle is mounted on the spray wand as the inoculum reservoir to shorten the fluid delivery tube. Periodically during spray application. the spray wand is raised vertically to shut off the spray, drain the delivery tube, and agitate the spore suspension.

VII. Requirements for the Infection Process

Successful artificial conditions for infection by the cereal rust pathogens should duplicate as nearly as possible the favorable natural conditions of temperature, light, moisture, and atmospheric purity. For most of the cereal rust pathogens, the entire infection process is completed in dew and darkness at a single optimum temperature. The infection process of *P. graminis*, however, is adapted to a diurnal pattern of temperature and light.

A. PHYSICAL FACTORS

Spores of *P. graminis* germinate and appressoria form on the moist leaf surface during darkness within the optimal temperature range of 15° to 24°C (Sharp *et al.*, 1958). If plants are then left under these conditions, only about 1% of the appressoria produce penetration pegs and substomatal vesicles. Most appressoria remain quiescent if left in dark, moist dew conditions or if dried slowly in dim light at a slightly lower temperature than that used during appressorial formation (Rowell *et al.*, 1958). Appressoria produce penetration structures if plants are kept wet with dew and the temperature is increased to 30°C either in darkness or with light greater than 5400 lux (Sharp *et al.*, 1958). Penetration also occurs from appressoria on plants dried slowly at about 3° to 5°C below that during the dew period temperature if the plants are then transferred to natural light at about 30°C in the glasshouse (Rowell *et al.*, 1958).

With *P. graminis*, infection rates under artificial conditions have never approached the expected maximum. Spore germination rates of 98 to 100% are possible in an oil drop on water agar, appressoria are produced consistently on plants by 50 to 60% of germinating spores under optimum conditions, and nearly all appressoria can penetrate plants under favorable field conditions. Thus infection rates of at least 50% would seem to be attainable. In practice, maximum infection rates of only 15 to 25% have been attainable. Germination on leaf surfaces of urediospores freshly collected from mature uredia generally is 10–20% less than that in an oil drop on water agar. This loss in germinability is not due to self-inhibitors, which do not inhibit germination at densities less than 7000 spores/cm^2 (Tollenaar and Houston, 1966), but may be due to the materials found by Woodbury and Stahmann (1970) to be associated with urediospores of *P. graminis* that form films on water surfaces and oxidize to form products inhibi-

tory to spore germination. It is also possible that nongerminating spores on the leaf surface failed to have contact with the free moisture that is essential for germ tube growth, because dew does not wet the entire leaf surface (Burrage, 1969). Although rates of appressorial formation under artificial conditions are comparable to those under natural conditions, the best rates of penetration achieved in trials, however, range between 30 and 40% of the appressoria produced. Thus either my artificial environments were suboptimal, or other factors were unfavorable for the completion of the penetration process.

The role of elevated temperatures and light in the completion of the infection process has not been resolved completely. As described by Staples and Macko (Chapter 9, this volume), infection structures have been induced to differentiate *in vitro* by exposure to 30°C without light (Maheshwari *et al.*, 1967), by a volatile substance present in steam distillates of urediospore extracts (Allen, 1957) thought to be acrolein (2-propenal) (Macko *et al.*, 1978), and by phenols extracted from epicuticular wax and cell walls combined with volatile fractions from wheat leaves (Grambow and Riedel, 1977). However, the appressoria induced by physical and chemical stimulation of germlings on aqueous media or polar membranes *in vitro* do not mimic exactly the structures formed on the wheat leaf. These methods of stimulating infection structures *in vitro* induce the formation of a roughly globular appressorium that immediately proceeds without interruption to produce the penetration structures. On the wheat leaf, however, the appressoria produced when a germ tube encounters stomata are elongated at right angles to the germ tube; they are tapered at the ends (roughly cigar-shaped) and are quiescent until stimulated to differentiate the penetration structures by exposure to light and elevated temperatures. When the regimen used *in vitro* to induce the formation of infection structures was used *in vivo* in dark dew chambers by first initiating spore germination on wheat leaves for a short period at 18°C and then transferring plants to 30°C, the penetration structures were differentiated on the external leaf surface (Sharp *et al.*, 1958). Thus the appressoria stimulated to form *in vitro* appear to be transitory, atypical structures formed in response to stimuli primarily required for the development of the penetration structures.

In contrast to the *in vitro* development of infection structures, *in vivo* development is enhanced by exposure to light after the appressoria are fully developed in the dark (Sharp *et al.*, 1958; Rowell *et al.*, 1958; Yirgou and Caldwell, 1968). Reduced penetration and infection of inoculated plants incubated in dark dew chambers does not appear to be due to physical exclusion by closed stomates. With *Puc-*

cinia recondita, exclusion of penetration structures by closed stomates results in deformation of appressoria and the formation of penetration structures on external host surfaces (Romig and Caldwell, 1964). With *P. graminis*, I have never observed distorted appressoria and rarely have seen penetration structures produced externally. Furthermore, Yirgou and Caldwell (1968) found that most stomates remain closed in normal and high light under appressoria and that most penetrated stomates were closed. They showed that high CO_2 concentrations inhibited penetration, which suggests that penetration is enhanced when CO_2 levels in the leaf are reduced by high photosynthetic activity in the light and inhibited by high respiratory CO_2 in the dark.

B. ATMOSPHERIC PURITY

Air pollutants can also reduce infection success of urediospore inoculum. Tobacco smoke inhibits the rate of germination and of germ tube growth (Melching *et al.*, 1974). Exposure of wheat seedlings to 0_3 prior to inoculation reduced the frequency of infection structures (Heagle and Key, 1973). Exposure to 0.1 ppm SO_2 either before or after inoculation seemed to reduce infection on Thatcher wheat, but infection on Prelude wheat was unaffected (Laurence *et al.*, 1979). The effect of the pollutant on the infection process during the dew period was not studied. Sharp (1972) found that urediospores of *Puccinia striiformis* germinated poorly in his laboratory during periods of high automotive traffic nearby when an atmospheric inversion existed, and the atmospheric concentration of large air ions and lead increased. Exposure to the pollutant during the first hour of spore hydration in the dew environment was the crucial time for inhibition.

C. DEW CHAMBER OPERATION

Programmable dew chambers were developed at the Cereal Rust Laboratory to provide a reproducible diurnal environment with dark and light phases at different temperatures for dependable incubation of seedling plants inoculated with *P. graminis*. The dew compartment is 1.52 m wide by 1.37 m long by 0.2 m high. Hot water at 32°C can be circulated in a pan beneath the plants, and coolant at 7°C can be circulated through copper coils in the top of the chamber. Cool white fluorescent lamps mounted on 19-cm centers beneath the cooling coils provide about 10,000 lux of light at the tips of the seedling leaves. The incubation cycle is started by placing inoculated seedlings in the

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stain should be freshly mixed for best results. The mixture is made from stock solutions as follows: 0.3 ml 2% acid fuchsin in ethyl alcohol, 0.3 ml 2% cotton blue in ethyl alcohol, 4.0 ml 1.24% acetic acid, 18.0 ml 95% ethyl alcohol, and 1 drop (0.05 ml) Tween 20 (polyethylene sorbitan monolaurate). The stain is applied with a Paasche H "3 in 1" Airbrush until the plant surface is coated evenly with fine droplets. The stain can be applied either to the intact plant or to a leaf segment mounted on a microscope slide by adhesive tape. The stained tissue is examined either in bright light from an epicondenser for thick, opaque tissues or from a substage condenser for thin tissues. Fungal protoplasts stain red, and walls stain blue. The extent of germ tube development, appressorial formation, and protoplast migration is readily apparent. This procedure is known not to disturb ungerminated spores on the host surface and can be used to determine spore deposition on leaf blades. The empty appressorium from which the protoplast has migrated into the substomatal vesicle indicates that the stomate has been penetrated. Some appressoria are observed to be partially empty, which presumably indicates that the penetration process was incomplete.

C. POSTPENETRATION DEVELOPMENT

Postpenetration development is observed readily in segments of seedling leaves by the method for fluorescence microscopy of Rohringer *et al.* (1977). In this method, infected tissue segments fixed in dilute lactophenol in ethanol are stained with an optical brightener (Calcofluor, American Cyanamid Co., Wayne, New Jersey) and then examined under incident ultraviolet light. The extent of pathogen development in the infection process from appressorium through the penetration peg, substomatal vesicle, infection hyphae, haustorial mother cells, secondary hyphae, sporophytic hyphae, and spore initials can be clearly traced. Mature urediospores do not fluoresce. Although haustoria are not visible in the preprations by this method, Kuck *et al.* (1981) have successfully stained haustoria by a modified procedure. Hypersensitively collapsed and necrotic host cells have a distinctive yellow-green fluorescence. Each necrotic cell is evident as a linear group of two to four fluorescing disks, each representing one of the two to four connected lobes of typical mesophyll cells. One can determine by this method the extent of development of each penetrant, and whether infection was established or the stage in the infection process at which development ceased (Rowell, 1981). Unfortunately, this method is ineffective for leaf blades from adult plants, presumably

because of the absorption of UV light by the heavy cuticle. Penetrant development in such adult tissues can be observed if the tissue fixed in lactophenol is cleared for several hours in saturated chloral hydrate and washed with water before further processing. The effective time for treating adult tissue in chloral hydrate varies with tissue age and cuticle thickness.

The method of McBride (1936) of clearing whole leaf segments with saturated chloral hydrate solution and staining with acid fuchsin is useful for observing haustoria (Leath and Rowell, 1966). The time required for clearing by this method can be reduced to about 24 hr by incubating samples at 60°C. Optimal clearing is critical for successful application of this method. Undercleared host tissue stains heavily with acid fuchsin, which obscures the fungal structures, whereas overcleared host tissue becomes very fragile and the fungal structures will not retain stain. Adult tissues do not clear readily by this method.

Growth of the rust fungus can be measured inside living leaves, although the method is laborious. With bright transmitted light at about ×400, hyphal tips at colony borders can be seen just beneath the epidermis in wet mounts (0.1% Tween 20) of seedling leaves. Apochromatic objective lenses are required with this method. Bushnell (1970) found a relatively constant growth rate of 1.1 mm/day in a compatible host–pathogen combination by repeatedly measuring the elongation of 8 to 10 colonies from first appearance of fleck to leaf senescence. Although the mature stem rust infection has a characteristic diamond shape, early colonial growth is highly variable in size and shape (Rowell, 1981). Some penetrants produce two infection hyphae, one at each end of the vesicle, but most penetrants produce only a single hypha. Growth of the infection hyphae and the initial secondary hyphae generally, but not invariably, is parallel to the long axis of the leaf. Thus early growth of the stem rust fungus in the host often is measured as the maximum extension of secondary hyphae into the host.

Although this measure may not accurately reflect the increase in mass of fungal tissue, it provides some perspective on the early rate of development of the rust pathogen in the host. In a study (Rowell, 1981) of postpenetration development of stem rust, my measurements of the maximum extension of secondary hyphae indicated that initial growth was very slow, about 58 μm/day, and the lag phase of growth extended for about 100 hr from inoculation (Fig. 2). Mayama *et al.* (1975) also found a prolonged lag phase in measuring growth of *P. graminis* by the glucosamine content of the infected susceptible host. In my study of 15 colonies/cm^2, the growth rate appeared to become constant after 113

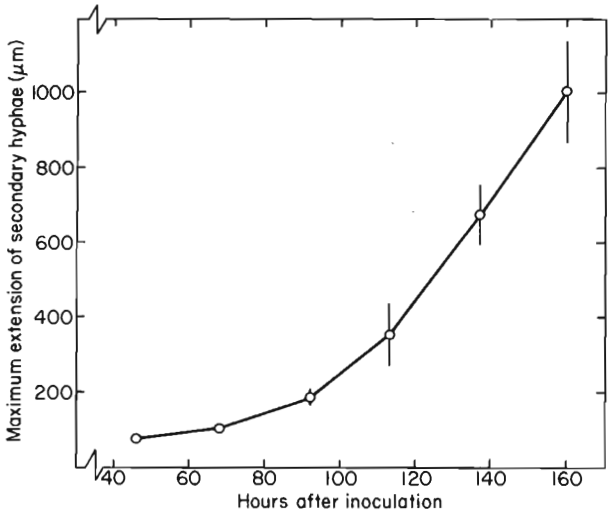

Fig. 2. Rate of spread of secondary hyphae of race 15B-TLM of *Puccinia graminis* f. sp. *tritici* into susceptible Baart wheat (J. B. Rowell, unpublished data). Vertical bars indicate one standard deviation on each side of means.

hr at about 360 μm/day, whereas Bushnell's measurements (1970) at lower rates of infection indicated that hyphal extension in a mature uredium was about 550 μm/day. Growth rates under crowded conditions are probably suppressed by intercolony competition for nutrients.

D. FREQUENCY OF UREDIA

Measuring rust infection in terms of number of uredia per unit area of the host requires reasonably accurate measurements of the area of host tissues. Ideally, the host tissue should be measured just prior to inoculation to eliminate errors due to subsequent elongation of leaves and stems.

Area measurements of host-tissues can be made indirectly by taping individual blades and stems on glass plates and preparing images on Ozalid paper (Higgins and Schreiber, 1954), or by reproducing the image on a photocopying machine. The surface area of the image is calculated from either weight or planimeter measurements. Unfortunately, this method destroys the host plant; therefore, a duplicate set of plants is required for measurement if the surface area is to be determined at the time of inoculation.

Equipment is available that directly measures leaf area. I have worked with the Model LI 3000 Portable Area Meter (Lambda Instruments Corp., Lincoln, Nebraska), which can measure rapidly the leaf area of intact wheat plants. The area measurements of a wheat seedling leaf with this instrument are reproducible within an error ≤5%, and the variation found between means of replicates each consisting of 24 leaves was ±3.8%. Contact with the scanning head before inoculation has had no effect on subsequent number of infections.

The number of uredia per square centimeter is not linearly proportional to the amount of inoculum at all dose levels, especially at higher levels (Fig. 3). In my experience with numerous inoculations of susceptible cultivars, the number of infections has been directly proportional to the amount of inoculum only up to about 10 uredia/cm^2 (~20–30 uredia per leaf); thereafter, the rate of increase in number of uredia declined. The decrease in efficiency of infection presumably results from competition and overgrowth between closely adjacent colonies. This presumption has not been tested, because the apparent decline in rate of infection occurred at colony densities too low for histological studies of spore deposition and the infection process. In my experimental work requiring controlled inoculation for comparison of treatments

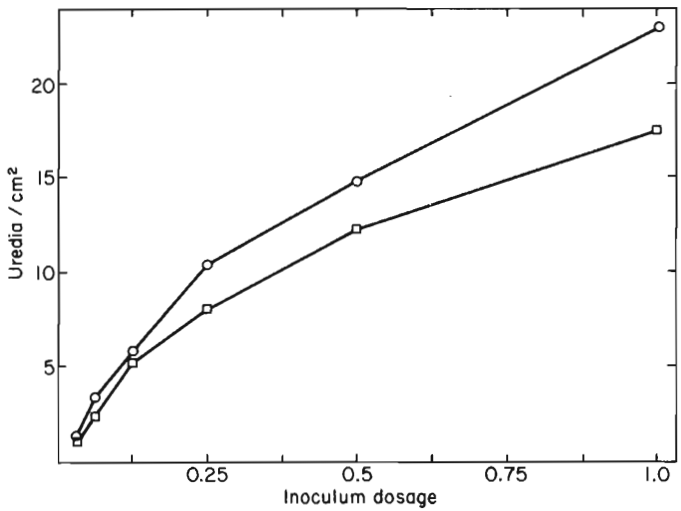

Fig. 3. Relation of inoculum dosage of race 15B-TLM of *Puccinia graminis* f. sp. *tritici* to the number of uredia produced on susceptible wheat cultivars Purdue 5481C1 (○) and Baart (□). [Inoculum dosage 1.0 = 4.25 mg of urediospores/ml in spray inoculum applied by a standard procedure (Rowell and Olien, 1957).] (J. B. Rowell, unpublished data.)

by the number of uredia produced, I used rates that yielded 10–20 uredia per leaf.

E. DISEASE SEVERITY ESTIMATES

When the number of uredia exceeds about 100 per leaf or plant, counting uredia on many samples is an extremely time-consuming method of data collection. Infection frequency under these conditions generally is estimated by using modified Cobb scales (Peterson *et al.*, 1948) or similar scales. The Cobb scale is based on the observation that the uredial area is about one-third of the total infected area. Thus at 100% severity only about one-third of the plant surface is occupied by uredia, but the host tissue is completely infected. The scale consists of a series of idealized diagrams that depict the frequencies of uredia for a series of percentages of disease severity. In practice, an investigator matches the frequency of uredia on the host with the appropriate diagram to determine the percentage severity. Counts of the number of uredia in each diagram yield a mean frequency of 0.35 uredia/cm^2 for 1% disease severity. The total surface area of blades, sheaths, and stems of a typical culm is about 150 cm^2; therefore 53 uredia would be equivalent to 1% severity (with leaf rust, which usually infects only leaf blades, 20 uredia would be equivalent to 1% severity for a typical plant with 56 cm^2 of leaf blade area). These frequencies are higher than that of 10 uredia per culm for 1% severity derived by Kingsolver *et al.* (1959) from counts of the number of uredia on wheat culms with different levels of estimated severity. For practical purposes, however, 10 uredia per culm for 1% severity is a satisfactory conversion factor because of the progressive changes with time in the amount of vulnerable host tissue.

X. Concluding Remarks

Success in the cooperative USDA–Minnesota program for breeding hard red spring wheat cultivars with enduring resistance to stem rust emerged as proficiency improved in producing infection under artificial conditions. When I started working with wheat stem rust in 1955, investigations on this disease in our glasshouses were suspended from late November until late February because of the difficulty of obtaining suitable levels of infection under the poor light conditions of win-

ter. Stem rust cultures were maintained by periodic transfers on susceptible hosts, which severely limited the size of culture collections. Enormous amounts of labor were used to initiate and develop severe epidemics in breeding nurseries. Rust spreader rows of susceptible cultivars for these nurseries were planted early in the growing season 4–6 weeks before test lines were planted, and repeatedly inoculated to assure abundant inoculum and heavy rust infection. This atypical late cultivation of the test wheats distorted the evaluation of the temperature-sensitive resistance conditioned by *Sr6,* which was considered ineffective under Minnesota growing conditions, a concern that was refuted by the performance of released cultivars such as Selkirk that possessed this resistance.

The efficiency and productivity of stem rust investigations were greatly increased by the progress made in developing long-term storage of rust spores, efficient inoculation techniques, improved dew chambers, effective environmental growth chambers, and adequate supplemental light in glasshouses. These improvements in methodology enabled large numbers of breeding lines to be tested intensively with numerous, pathogenically diverse rust cultures in the glasshouse as well as in the field. The increased effectiveness of the tests is evident in the endurance of the resistant hard red spring wheat cultivars released by the Minnesota program since 1955, none of which has succumbed to stem rust. This program also produced about 80% of the wheat lines identified as elite germ plasm for stem rust resistance in tests in the Uniform and International Spring Wheat Rust Nurseries. These achievements testify to the importance of the improved techniques now available for working with wheat stem rust.

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