HOST-PATHOGEN INTERACTIONS BETWEEN WILD RICE (*Zizania aquatica* L.) AND *Helminthosporium oryzae* Breda de Haan.

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by

Linda Mary MacKenzie

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ABSTRACT

Comparisons were made between healthy and Helminthosporium oryzae-infected wild rice aerial and floating leaves using bright field, epifluorescence and transmission electron microscopy.

Spore germination and penetration was similar on both leaf types, however the duration of these stages was longer on the floating leaves. Affected host cells on both aerial and floating leaves exhibited a characteristic bright yellow autofluorescence which was quenched by brown pigments as final lesion color was achieved. H. oryzae progressed intracellularly in vascular bundles and bundle sheath cells and intercellularly in areas of mesophyll cells; at the cellular level, the pathogen destroyed mesophyll cell walls and organelles. Non-invaded epidermal cells appeared to remain intact, with only some conformational wall changes and swelling. These stages were examined using epifluorescence microscopy in conjunction with the fluorochromes Calcofluor and ethidium bromide which effectively distinguished H. oryzae infection structures. Correlations between this technique and bright field microscopy using methylene blue to discern infection structures and host necrosis indicated that the characteristic yellow autofluorescence of host cells was due to the build up of phenolics during lesion formation. In addition, sporulation studies indicated that floating leaves may be capable of providing a potential source of inoculum prior to outbreaks of Helminthosporium blight of wild rice. Floating leaves may also provide plant breeders with an alternate system for screening wild rice varieties for pathogen resistance.

The interaction of wild rice pollen with germ tubes of H. oryzae indicated that the presence of pollen may increase disease severity.

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ABBREVIATIONS

AB	aniline blue
AF	acid fuchsin
AP	appressorium
BC	bulliform cell
BCB	bulliform cell band
BRL	brown-ringed lesion
C	Calcofluor
CFH	Calcofluor-fluorescent hyphae
CH	chloroplast
CL	coalesced lesion
CW	cell wall
DL	discrete lesion
EB	ethidium bromide
FC	fusoid cell
G	guard cell
GHC	granular host cytoplasm
HDL	haloed discrete lesion
HL	haloed lesion
IBS	inner bundle sheath
L	lipid body
LC	long cell
M	mitochondrion
MB	methylene blue
MY	microbody
N	nucleus
OBS	outer bundle sheath
OW	outer cell wall
P	parenchyma cell
PA	papilla
PD	plasmodesmata
PH	phloem
p.i.	post-inoculation
PM	plasmamembrane
S	stomate
SB	stomatal band
SC	sclerenchyma cell
Si	silica
ST	starch
SU	subsidiary cell
TB	toluidine blue
TH	thylakoid
TV	transverse diaphragm
V	vein
X	xylem

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INTRODUCTION

Wild rice (Zizania aquatica L.) is an annual aquatic cereal, native to the shallow lakes and streams of Manitoba, and other parts of eastern temperate North America. Traditionally, wild rice from natural stands was a major food for Indian tribes, but its recent increase in commercial importance comes largely from developments in Minnesota in the 1960's. There, growers were able to increase overall production by employing paddy-culture techniques and combine harvesting. Nevertheless yields of individual paddies have suffered major fluctuations because of depredation by pests and diseases.

One of the most important diseases of wild rice is brown spotting and necrosis of the leaves, caused mainly by Helminthosporium and Fusarium species. Kernkamp et al. (1976) found that in cultivated fields Helminthosporium blight usually occurred at low levels in the first year and then increased in severity each year to the point where crops were completely destroyed by the third or subsequent years. Although severe blight epidemics of this kind have not been observed in Minnesota's natural stands, Stone et al. (1975) reported that natural stand yields in Manitoba could be reduced considerably by this disease.

It is not surprising, in the light of the very recent domestication of wild rice, that little is known of symptom development and pathological changes induced as a result of infection by Helminthosporium oryzae Breda de Haan. Studies of other potential pathogens of wild rice have generally been limited to identification and distribution (Gilbert, 1974; McQueen, 1981), although the latter author has

suggested that pathogens of aerial leaves may also infect floating leaves with a resultant loss in yield, or in severe cases, plant death. The role of the floating leaves as a source of inoculum for the severe, often sudden, Helminthosporium blight epidemics is poorly understood. An elucidation of the infection stage and early histopathology of Helminthosporium blight disease is an essential prelude to epidemiological studies and to control by breeding and chemical means. The basis for studying symptom development and pathological change induced by foliar pathogens, such as H. oryzae, is an understanding of the host anatomy and morphology. However, the few investigations of wild rice which have been reported have concentrated on the taxonomic characters of the aerial leaves.

The presence of pollen at the time of infection is known to increase the incidence of some diseases and Gilbert (1974) has suggested that this is true of wild rice pollen and Helminthosporium blight. He also suggested that algal blooms affected wild rice at the floating leaf stage. Photomicrography of pollen or algal interactions with foliar pathogens is limited.

Because of the limited information available on Helminthosporium oryzae blight of wild rice, a study was conducted whose principal objectives were:

1) to develop appropriate techniques, to compare the anatomy of healthy and H. oryzae-infected aerial leaves of wild rice and to trace the development of the pathogen in the latter;
2) to study the anatomy of healthy floating leaves and those infected with H. oryzae and to determine the conditions under which floating leaf lesions would provide inoculum for aerial leaf

infections;

3) to study the nature of the interaction between H. oryzae and both wild rice pollen (aerial leaves) and cyanophyte algae (floating leaves) during the infection process.

LITERATURE REVIEW

Zizania aquatica L. - the host

The most comprehensive summary of the botany, distribution, and utilization of American wild rice was given by Dore (1969); however, neither he nor other authors have provided a systematic anatomical account of this species. Weir and Dale (1960) compared some of the anatomical characteristics of the three wild rice leaf types - submerged, floating and aerial - but their main emphasis was on embryo development and plant life history. They noted major epidermal cell differences which distinguished the three leaf types. The ultrastructure of wild rice leaf surfaces, in particular the epicuticular wax was examined by Hawthorn and Stewart (1970), who found that factors other than the day-night cycle were responsible for the wax ultrastructure and suggested that these wax forms may significantly affect the survival of wild rice. In a taxonomic study of *Zizania*, Terrell and Wergin (1979) noted major similarities between the aerial leaf abaxial surfaces of the four species.

Comparisons have been made between *Zizania* sp. and *Oryza* sp. with regard to leaf anatomy and morphology; those for *Z. latifolia* (Griseb.) Turcz and *O. sativa* L. were summarized by Metcalfe (1960). Terrell and Robinson (1974) found both genera to have arm-type parenchyma cells, a complex system of vascular bundles in the mid-rib region, Oryza-type silica bodies, and large, overarching papillae which nearly conceal the stomata. Other anatomical research using conventional microscopy was done by Holm (1896), Tullis (1935), and Kaufman (1959), while Hau and Rush (1982) examined the leaf morphology by means of a scanning electron microscope.

Gilbert (1974) conducted the first extensive study of foliar pathogens of wild rice and found Entyloma lineatum (Cke.) J.J. Davis, Alternaria and Cladosporium isolates to be weakly pathogenic. In Minnesota, Kernkamp et al. (1976) found stem rot caused by Helminthosporium sigmoideum Cav. and Sclerotium spp., ergot caused by Claviceps zizaniae (Fyles) Pantidou, and stem and leaf smut caused by E. lineatum. In Manitoba, McQueen (1981) reported leaf and stem smut, as well as ergot, and in addition identified two new diseases associated with floating leaves, anthracnose caused by Colletotrichum sp. and leaf blotch by an unidentified pycnidial fungus. Three new diseases have been reported in Minnesota; zonate eyespot (Kardin et al., 1981), bacterial leaf streak caused by Pseudomonas syringae Van Hall (Bowden and Percich, 1891), and wheat streak mosaic, the only virus disease yet reported on wild rice (Berger et al., 1981). However, the most devastating disease of wild rice in natural stands and in paddies is the brown spot syndrome caused by Helminthosporium oryzae Breda de Haan, H. sativum Pam., King & Bakke and Fusarium spp. (Gilbert, 1974; McQueen, 1981), which was found to cause considerable reduction in yields in Manitoba (Rogalsky et al., 1971; Gilbert, 1974; Stone et al., 1975), and also in Minnesota (Bean and Schwartz, 1961; Kernkamp et al., 1976). Kernkamp et al. (1976) segregated the causal organisms H. oryzae and H. sativum from the others and referred to the disease as Helminthosporium blight, while noting severe epidemics of blight in paddies but not in native Minnesota stands. Wild rice breeding research has focused on developing varieties resistant to Helminthosporium blight and combining these with varieties showing desirable traits of early maturity and shattering resistance (Elliott, 1975; 1977; Elliott and Perlinger, 1977).

Helminthosporium oryzae - the pathogen

Helminthosporium oryzae Breda de Haan is the conidial state of *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechsler ex Dastur. *H. oryzae* was transferred to the genus *Drechslera* as *Drechslera oryzae* (Breda de Haan) Subram. & Jain (Ellis and Holliday, 1971). However, the name *H. oryzae* is most commonly used in the literature.

Although strains of *H. oryzae* exist which differ morphologically and physiologically, there is apparently no specialization in the form of race differentiation (Ellis and Holliday, 1971). Three isolates of the pathogen from *Z. aquatica* were pathogenic to *Oryza sativa* and *Z. latifolia* (Chang 1974b;1975). Chang (1978) found a strain with slender elongate conidia from rice-leaf brown-spot which was pathogenic to both *O. sativa* and *Z. latifolia*. Although various grasses can be infected by artificial inoculation, under natural conditions the pathogen attacks only *Oryza*, *Zizania* and *Leersia hexandra* Sw. (Chattopadhyay and Chakrabarti, 1953; Ou, 1972; Chiu *et al.*, 1972; Chang, 1975). The leaf morphology of these hosts is very similar (Metcalfe, 1960).

H. oryzae has been found to produce the phytotoxin, ophiobolin (Lindberg, 1971; Ou, 1972). Ophiobolin was detected at the site of fungal invasion on rice leaves and typical disease symptoms were induced (Narain and Simhachalam, 1975). Symptom induction was correlated to phytotoxin presence by assaying extracts collected from *H. oryzae*-infected leaf tissue in the latter study. Chattopadhyay and Samaddar (1976) found that ophiobolin caused permeability changes of a non-specific nature in rice tissues and suggested that the cell membranes were rapidly disrupted as a result of contact with the phytotoxin.

Intermittent illumination is needed to trigger sporulation of H. oryzae (Chang, 1974a; Sharma and Singh, 1975; Hau and Rush, 1980), the optimum temperature for sporulation being 27-28°C (Gilbert, 1974; Hau and Rush, 1980). Growth substrates containing plant parts produced the most abundant sporulation, however, many other natural and synthetic media have been used successfully with particular isolates (Sharma and Singh, 1975; Chang, 1980; Hau and Rush, 1980).

Factors which promoted infectivity of H. oryzae have been reported: (1) temperature in the 20-30°C range; (2) relative humidity above 95%; (3) presence of water on the leaves prior to inoculation; (4) evening inoculation; and (5) leaf exudates, which stimulated germ tube growth significantly (Sherf et al., 1947; Gilbert, 1974; Purkayastha and Mukhopadhyay, 1974; Sarkar and Sen Gupta, 1977). Page et al. (1947) studied the effect of temperature and relative humidity on the longevity of the conidia of H. oryzae and found that warm, moist climates were unfavorable to longevity under field conditions.

Host-pathogen interactions

While Kernkamp et al. (1976) described the symptoms and part of the Helminthosporium blight disease cycle, the only microscopic study of a host-pathogen interaction related to wild rice is an observation of the preliminary stages of H. sativum infection (Gousseau, 1979).

In contrast, the disease cycle and pathophysiology of the H. oryzae-O. sativa interaction have been extensively investigated (Ou, 1972), and certain of these results are pertinent here. Earlier reports of the morphology of preinfectional interactions were confirmed by Locci (1969) and Hau and Rush (1979;1982), who described appressorium formation

and noted that an extracellular mucilaginous sheath adhered to wax crystals which possibly enabled the fungus to penetrate the leaf surface. Tullis (1935) observed both intercellular and intracellular invasion by H. oryzae hyphae in differing areas of the mesophyll of the host; he reported yellow brown deposits of an unknown chemical nature associated with the infection. Horino and Akai (1968) found that after fungal hyphae had penetrated the middle layer of radial walls of epidermal cells and intruded into the cells, substances accumulated due to cellular degeneration. Horino (1973) reported fungal-induced morphological changes in nuclei, mitochondria and chloroplasts, as well as an accumulation of lipid and starch. He also suggested that the observed vesicles were degradation debris from chloroplasts and mitochondria. Sridhar et al. (1973) reported lipid discoloration in the early stages of disease development. Sporulation of H. oryzae on rice leaves was observed by Fukatsu and Kakizaki (1955), and the stages were described in detail. Matsuura (1973) found that sporulation coincided with an exhaustion of nutrients available to the mycelium. Lam and Thrower (1973) found that H. oryzae hyphal fragments infected rice plants in a manner comparable to conidia.

Infection of rice leaves by H. oryzae causes changes in the phenolic content of host tissue, which may be related to symptom expression. Oku (1958) noted high activity of polyphenol oxidase (PPO) in H. oryzae and low activity of this enzyme in rice leaves. PPO was found to oxidize several plant polyphenols giving rise to highly toxic substances (Oku, 1958; 1960a). Oku (1960a; 1960b; 1962) hypothesized that fungal infection caused an increase in phenolics followed by the

formation of quinones by enzymatic oxidation resulting in the formation of brown necrotic lesions. Subsequently, Kitani et al. (1972) found increased levels of flavonoids and phenol carbonic acid and suggested that the increase in peroxidase activity observed in lesions might inhibit lesion formation, while Shishiyama et al. (1973) suggested that β-D-glycosidase synthesized in infected rice leaves may release phenolic compounds from their glycosides. Confirmation of the suggested accumulation of phenols and PPO activity was given by Chattopadhyay and Bera (1980), who suggested that increased phenol content might arise either from healthy tissue in response to infection or from the release of bound phenolics by pathogen enzymes; PPO had either a host or pathogen source. The culmination of this research was an hypothesis for the development of brown spot symptoms based on that of Oku (1962), but which takes into account the influence of the H. oryzae phytotoxin, ophiobolin. Chattopadhyay and Samaddar (1980a) reported that the physiological changes observed during disease development, such as membrane disruption and changes in respiration, protein synthesis and nucleic acid synthesis might be due to the synthetic activity of the fungus in that these effects are also produced by ophiobolin. Chattopadhyay and Samaddar (1980b) proposed that: (1) following ingress the pathogen produces both ophiobolin and PPO; (2) ophiobolin rapidly disrupts cellular metabolism leading to cell death; (3) bound PPO and phenolics are released from such moribund cells; (4) and the released phenolics are then oxidized to quinones and finally to brown pigments by PPO of both pathogen and host origin.

Host cells affected by pathogens have been shown to take on a

bright primary gold to yellow autofluorescence in response to u.v. illumination. This is true of leaf tissues responding hypersensitively to infection by rust and powdery mildew fungi (Marte and Montalbini, 1972; Tani et al., 1975; Mayama and Shishiyama, 1976a;1976b; Rohringer et al., 1977; Samborski et al., 1977; Koga et al., 1980; Kidger and Carver, 1981). Marte and Montalbini (1972) concluded that yellow fluorescence, one of the earliest detectable signs of alteration of host cells, coincided with phenol accumulation and disappeared with the formation of brown pigmentation. That the autofluorescent and u.v. absorbing substances which accumulated were polyphenol compounds was confirmed by Mayama and Shishiyama (1978) and Kita et al. (1980). Koga et al. (1980) suggested that fluorescent chemicals deposited at the infection sites might represent chemical factors which are associated with resistance expressed at the penetration stage, while Kita et al. (1981) proposed that incompatibility was expressed by fluorescence of epidermal cytoplasm. Yellow autofluorescence of H. sativum-affected wheat leaf cells indicated that this is not a specific hypersensitive response to rust and powdery mildew fungi (Stockwell and Sherwood, 1981). Although other Helminthosporium spp., such as H. maydis Nisikado & Miyake and H. carbonum Ullstrup, have been shown to produce phytotoxins (Heitefuss and Williams, 1976), there have been no reports of yellow autofluorescence of host tissues in response to invasion by them. However, reports on the development of H. carbonum, H. maydis, and H. sorokinianum Sacc. ex Sorok. in their respective hosts indicated that invasion was correlated with host necrosis (Jennings and Ullstrup, 1957; Healy and Britton, 1968; Murray and Maxwell, 1975).

Microscopy and Microtechniques

Drawbacks in conventional whole leaf clearing methods (Clarke, 1960; Shipton and Brown, 1962; Skipp and Samborski, 1974) resulted in the development of techniques involving epifluorescence microscopy by Rohringer (1977), who noted that these techniques heightened the differentiation and allowed the visualization of the leaf surface, fungal infection structures, and affected host cells. Various fluorochromes have been used in conjunction with epifluorescence microscopy in the study of plant structures and host-pathogen interactions.

Calcofluor, an optical brightener, is a fluorochrome of the diamino-stilbene-disulphonate type which has been found to bind to cellulose, chitin, and other β -linked polymers (Maeda and Ishida, 1967; Harrington and Raper, 1968; Hayashibe and Katohda, 1973). Calcofluor-treated higher plant cell walls fluoresced brilliant blue, while most cytoplasmic components of these cells were normally unstained (Hughes and McCully, 1975; Peterson et al., 1978; Peterson and Peterson, 1980). Tsao (1970) studied spore germination, mycelial growth, sporulation and lysis of Phytophthora parasitica Dast. and several other soil fungi. Since then it has also been used to distinguish infection structures of various rust fungi (Patton and Johnson, 1970; Rohringer et al., 1977; Samborski et al., 1977; Kuck et al., 1981), and Helminthosporium sativum (Stockwell and Sherwood, 1981).

Ethidium bromide (EB) is a heterocyclic organic compound of the phenanthroline group which binds to both DNA (Elliott, 1963; LePecq and Paoletti, 1966; 1967; LePecq, 1971) and RNA (Burns, 1972). EB has been used in the study of plant structure and O'Brien and McCully (1981)

noted that EB binds strongly to lignified walls and weakly to plant cytoplasm. EB imparts a characteristic orange-red fluorescence to fungal hyphae (Calich et al., 1978; Roser, 1980; Kuck et al., 1981; Roser et al., 1982).

Smith and McCully (1978a) suggested that the fluorescence induced by aniline blue (AB) did not specifically indicate the presence of β-1,3-glucans and that most wall structures fluoresced to some extent after application of the fluorochrome. This contradicted earlier work (Eschrich and Currier, 1964) and confirmed observations by Faulkner et al. (1973) that AB was not specific for any one type of isolated glucan. Plant structures were often both autofluorescent and AB fluorescent and AB fluorescence was modified by lignins and phenolics (Smith and McCully, 1978a). AB has been widely used in botanical microtechnique (Currier and Strugger, 1956; Dionne and Spicer, 1958; Martin, 1959; Jeffries and Belcher, 1974; Polito and Luza, 1981).

Acridine orange has been used to study host-pathogen interactions by Kunoh and Ishizaki (1981). They observed that this fluorochrome was translocated between conidia of E. graminis DC ex Merat and infected barley cells via pathogen germ tubes.

Bennett et al. (1976) reviewed the current techniques for preparing plastic-embedded tissues for light microscopy and discussed suitable conventional staining techniques. Stains of this nature which have been successfully used to stain pathogens on or in host tissues include: methylene blue (Tullis, 1935; Buczacki and Moxham, 1979; Alexander and Bigg, 1981); toluidine blue (Lazarovits and Higgins, 1976a; 1976b; Gladders and Coley-Smith, 1979; Mansfield and Richardson, 1981); and trypan blue (Boedijn, 1956; Healy and Britton, 1968; Gladders and

Coley-Smith, 1979).

Special electron microscopy techniques have been designed for use with delicate tissues. Fowke (1975) described a procedure for examining technique-sensitive protoplasts, which was modifed by Mills and Chong (1977) for a study of rapeseed. Harder et al. (1978) then used the modifed procedure to study the ultrastructure of stem rust of wheat. Current concepts in plant ultrastructure have been reviewed and illustrated by Ledbetter and Porter (1970) and Gunning and Steer (1975).

Pollen-pathogen interactions

The interaction of pollen grains with pathogenic fungi on leaf surfaces often increased the severity of the disease on the host plant, and suggestions have been made that it was the presence of pollen at the early stages of infection which stimulated fungal growth, thus contributing indirectly to an increase in disease incidence. Pollen grains of almond (Ogawa and English, 1960), American Holly (Bachelder and Orton, 1963), strawberry and bean (Chou and Preece, 1968), as well as other plants (Borecka and Millikan, 1973) enhance infections of Botrytis cinerea Fr. via stimulatory substances which induce more rapid spore germination and germ tube growth. Chou and Preece (1968) suggested the active principle from the pollen grains of bean and strawberry was water-soluble, dialysable and heat stable, and suggested that substances other than sugars were involved. Moreover, Vicia faba pollen when present enabled B. cinerea to be transformed from a mildly aggressive to a very destructive pathogen (Chou and Preece, 1968); the presence of the pollen enabled B. cinerea to overcome the inhibitory action of the phytoalexin wyerone acid (Mansfield and Deverall, 1971).

The nature of the stimulatory factor(s) of pollen has been investigated. Warren (1972a;1972b) found that only mixtures of sugars with boric acid reproduced the effect of sugarbeet pollen on both numbers and size of expanding lesions induced by Phoma betae Frank; however he doubted that the pollen factor corresponded to that described by Chou (1970) (cf Warren, 1972b) as the borate ion is anionic while the pollen factor described by Chou was in the cationic fraction. In a series of papers, Strange and his co-workers reported on the interaction between wheat anther and pollen extracts and Fusarium spp. (Strange and Smith, 1970; 1971a; 1971b; 1978a; 1978b; Strange et al., 1972; 1974; 1978). They suggested that choline and betaine were fungal growth stimulants and that the requirement of certain Fusarium spp. for these substances arose possibly from a lack of membrane precursors, but that some other process could also be involved for which these compounds served as triggers. As only a few Fusarium spp. responded to these compounds, they concluded that the phenomenon was far from universal amongst fungi. Choline and betaine were considered determinants of susceptibility in that they increased growth of the parasite in vivo. Lastly, Strange and his co-workers proposed eight principles which must be determined with regard to the factors in pollen responsible for stimulant activity.

In 1971, Fokkema did an extensive study on the effect of pollen in the phylloplane of rye on infection by Helminthosporium sativum. He found that infection was greatly stimulated if either pollen or pollen diffusate were added at the moment of inoculation. Further growth was enhanced by the presence of pollen, suggesting that this could increase the number of infection sites, in turn increasing necrosis.

The addition of pollen to leaves lacking a natural pollen deposit, and consequently with a less well developed microflora, caused a subsequent marked stimulation of infection by H. sativum. Barash et al. (1964) had found that leachates from safflower blossoms contained pectic substances which induced the production of polygalacturonase in germinating conidia of B. cinerea. Similarly, Fokkema found that pollen diffusate added to the fungal growth medium resulted in a distinct increase in cellulase and polygalacturonase production. The induction of these enzymes by host material (pollen), prior to penetration and the initiation of the defence mechanism of the host, may be of great advantage to the pathogen.

Preliminary observations suggest that an association of H. oryzae and wild rice pollen on aerial leaves of wild rice could increase the severity of disease. Gilbert (1974) felt that such a pollen effect could help to account for the heavy infection by Dreschlera isolates, particularly in the case of the second inoculations which were carried out at the time of flowering. Moreover, epidemics of Helminthosporium blight of wild rice usually occurred when the plants were in flower (Kernkamp et al., 1976).

Hawthorn (1968) reported that algae caused wild rice death at the floating leaf stage by adhering to the surfaces of the leaves and bleaching them. It was also suggested that algal blooms reduce light availability to seedlings by forming mats on the water surface (Gilbert, 1974). The effect of damage to leaves by algae in conjunction with fungal pathogens has not, to this author's knowledge, been reported in the literature.

METHODS AND MATERIALS

A. Pathological Techniques

1. Wild rice culture

Wild rice (Zizania aquatica L.) seed was originally obtained from Lac du Bois, Manitoba (50°18"N; 19°43"W). Following after-ripening for 90 days at 4°C in distilled H_2O in the dark, seed was germinated in fresh distilled water by gradually allowing it to warm to room temperature (21°C). A 12 h photoperiod (100 watt incandescent) was maintained for five to eight days until the germinating seedlings had produced two to three submersed leaves.

Seedlings were transferred to the greenhouse, planted in 6 inches of a mixture of sterilized black soil, sand and peat moss (2:1:1) in 4L plastic pails which were then flooded with tap water. Cool white fluorescent lights (CW General Electric) giving a 14 h photoperiod were suspended about 20 cm from the pots and gradually raised as the plants grew. Greenhouse temperatures throughout the 12 weeks of growth were maintained at 20°C (day) / 16°C (night) with relative humidity (RH) kept above 50%, using plastic sheeting to cover pots when necessary. Algal accumulations were washed off the water surface to keep the water clear.

Plants produced in this manner were allowed to cross-fertilize and the seeds produced were harvested by hand over a period of about three weeks. The daily harvest of seeds was immediately placed in distilled water and stored at 4°C for approximately 90 days. These seeds then formed a stock of uniform origin for all further experiments involving the use of wild rice floating and aerial leaves.

All wild rice stock seeds were germinated on a layer of sand in 4L of distilled water, as described above. For aerial leaf experiments, seedlings were grown in the greenhouse using the conditions and techniques described above, for a period of approximately 12 weeks. They were then transferred to a Model EF 7 (Controlled Environments Ltd., Winnipeg, Manitoba) growth chamber. Growth chamber conditions were 22°C (day) / 18°C (night) temperature, Gro-lux 14 h illumination (138.0 watts/m^2), and relative humidity (RH) 90% for one week prior to pre-disposition (see part 3 - inoculation procedures). For floating leaf experiments, seedlings were planted in soil mixture in styrofoam tubs flooded with distilled water, and placed in the Model EF 7 growth chamber, using conditions described for aerial leaves. Algal accumulation was gently hosed off leaves when necessary with distilled water at ambient temperature. After 3-4 weeks, when the second floating leaf began to extend, seedlings were considered ready for inoculation.

2. Fungal culture

For sporulation studies, thirteen isolates of Helminthosporium oryzae Breda de Haan in the University of Manitoba collection were tested for ability to sporulate in culture. Three single spore lines of each isolate were established in petri dishes on six growth substrates - autoclaved aerial leaves (AAL), autoclaved floating leaves (AFL), AAL in Sach's Agar, AFL in Sach's Agar, Sach's agar, and filter paper saturated with liquid Sach's solution. The AAL (3 x 4cm^2 pieces) and AFL (6 x 2cm^2 pieces) substrates were placed on moistened filter paper. The isolates were grown for 14 days at 25°C using a 12 h photoperiod (F20T12 BLB Blacklight General Electric/ F20T12 CW Vigorlight GE).

For infection studies, only H. oryzae isolate FA 72 D#5, isolated

from wild rice in Fort Alexander, Manitoba was used. This isolate was grown on autoclaved aerial leaves placed on moist sterilized filter paper in glass petri dishes using the afore-mentioned light and temperature regimes.

3. Inoculation procedures

Two days prior to inoculation, plants with both leaf types were pre-disposed in the growth chamber. For aerial leaf experiments, the temperature was raised to 22°C (day) / 20°C (night) and RH increased to 95-100% supplemented by a DeVilbiss humidifier in the chamber. For floating leaf experiments, the RH was increased to 100%; however the supplementary humidification was not required. Light conditions were unchanged.

H. oryzae spores were harvested under aseptic conditions. The filter paper and autoclaved leaf pieces in the petri plates were saturated with 10 to 15 mL of double distilled H_2O in which 1 drop of Tween 20/ 100 mL had been dissolved. The leaf pieces were then gently agitated with a wire loop, and the resulting spore suspension was adjusted to 5.0×10^4 spores/mL by replicated haemocytometer counts and serial dilution. The final spore suspension was used for spray inoculation with the inoculator held approximately 30 cm from both leaf types.

Fifty plants with 2 floating leaves each were inoculated with the spore suspension: ten plants protected with plastic sheeting during the inoculation served as controls. All exposed surfaces of the floating leaves received 25 mL or 50 mL per 25 plants. Depending on the experiment (see Part B - microtechniques), leaf samples were removed at the appropriate time. At 50 h post-inoculation (p.i.) any plants

with leaves remaining were returned to pre-inoculation growth chamber conditions for approximately one week. These plants were then transferred to the greenhouse and exposed to standard aerial leaf conditions to await sporulation of the pathogen on the leaves.

Inoculations were carried out on both attached as well as detached aerial leaves. In five replicate experiments, ten plants, each with five aerial leaves per plant were randomly sprayed with 10 mL of the final spore suspension. Natural pollen deposit was allowed to remain and no pollen was added. In each experiment, one plant serving as a control, was removed prior to inoculation and returned immediately thereafter. Depending on the type of experiment, (see Part B-microtechniques) leaf samples were removed at the appropriate time.

Aerial leaves were detached from the parent plant and cut into 4.0 cm^2 segments. These detached segments were placed in glass petri plates lined with sterile filter paper (4 segments for each of 5 plants) and floated on 0.1 g/L 2-aminobenzimidazole in sterilized distilled water. The contents of each plate were sprayed with 5 mL of the spore suspension and the plates were sealed with masking tape. Such plates were incubated with the corresponding inoculated plants in the growth chamber. Infected attached aerial leaves were simultaneously compared with detached leaf segments when studying lesion development.

At 50 h p.i., conditions in the growth chamber were restored to those used prior to inoculation and pre-disposition, that is, 20°C (day) / 16°C (night) temperature and RH of 90%. After 7 days in the growth chamber, any remaining infected plants were returned to the greenhouse and observed until sporulation of H. oryzae occurred at

approximately 10 weeks p.i. In addition, aerial leaves infected with H. oryzae which developed lesions were removed from plants 25 days p.i., pressed dry and stored. In order to maintain pathogenicity in each suceeding experiment, fungal isolate FA 72 D#5 was reisolated from these dried leaves and single spore lines were maintained for harvesting.

B. Microscopy and Microtechnique

1. Whole leaf mounts

To study the infection process, aerial leaf samples were removed at 18, 22, 24, 26, 30, 33, and 36 hours post-inoculation; floating leaf samples were taken at 48 hours post-inoculation. All leaves were fixed and cleared by boiling for 1.5 min in lactophenol/ethanol (1:2 v/v) and stored overnight in this mixture at room temperature. Then one of two preparative techniques for epifluorescence microscopy was employed for all leaf samples. Cleared specimens were washed twice for 15 min each with 50% ethanol, twice for 15 min each with 0.05M NaOH, three times with water, and then placed in 0.1M TRIS/HCl buffer, pH 8.5 for 30 min. They were treated for 5 min with a 0.1% solution of Calcofluor M2R New in this buffer. This was followed by washing four times with water (10 min each) and finally with 25% aqueous glycerol for 30 min. The specimens were mounted in glycerol containing a trace of lactophenol as a preservative and were ready for examination (Rohringer et al., 1977).

Alternatively, cleared specimens were washed twice for 15 min each with 50% ethanol and then stored at 4°C in 50% ethanol. After storage specimens were allowed to warm to room temperature, washed in double distilled H_2O for approximately 2 min, treated for 2 min with the fluorochrome of choice, then washed again for 1-2 min prior to mounting in double distilled H_2O on a slide for examination. Fluorochromes applied in this way were Calcofluor White M2R (0.1% in 0.1M TRIS/HCl, ph 8.5), aniline blue (0.001% in 0.03M Na_2CO_3), ethidium bromide (0.001% in 0.03M phosphate buffer, pH 7.0; or 0.005% in 0.05M phosphate buffer

pH 7.0), Coriphosphene O (0.001%), Nile Blue A (0.001%), primuline (0.001%), congo red (0.001%), Rhodamine B (0.001% aq.), Auramine O (0.001%), acriflavine (0.001%), and picridine orange (0.001%).

Leaf material was examined using a Zeiss Research Microscope equipped with Zeiss epifluorescence equipment NXL. The epifluorescence system had an HBO 50 light source and red suppression filter BG 38. The exciter and barrier filter combinations described below are referred to in the text and captions by the code designation:

Filter System Code	Exciter (nm)	Barrier (nm)
F1-I	390-440	475
F1-II	450-500	528
F2-I	365	418
F2-II	450-490	520
F3-I	405	418
F3-II	546	590

Three types of film - KODAK Ektachrome 64, Kodacolor II and Ektachrome 160 tungsten corrected - were used in conjunction with a KODAK blue filter #LB-45 inserted between the microscope tube and the camera attachment.

A WILD LEITZ M-8 Zoom Stereo dissecting microscope and camera unit were used to photograph sporulation of the pathogen on wild rice floating leaves.

2. Thin sections

Aerial leaf samples were removed at 18, 22, 24, 26, 30, 33, 36, 48, 72, and 96 hours post-inoculation. Floating leaf specimens were removed at 48 hours post-inoculation. For plastic embedding, specimens

were fixed for 2 h in 1.5% glutaraldehyde in 0.02M phosphate buffer, pH 6.8 with 0.75% acrolein added, and then transferred to a 6% glutaraldehyde in 0.025M phosphate buffer (pH 6.8) solution overnight at 4°C. Fixed specimens were washed twice in 0.025M phosphate buffer for 30 min each and dehydrated at 4°C in 2 changes of 2-methoxyethanol over 24 h. Final dehydration was carried out with ethanol, propanol and butanol (Feder and O'Brien, 1968). Dehydrated leaf samples were stored in butanol at -20°C until needed, and then infiltrated either with JB-4 Embedding Kit (Polysciences, Inc., Warrington, Pa) or glycol methacrylate (GMA).

The JB-4 plastic procedure was modified in that infiltration was overnight on a rotator at room temperature and embedding was for 2 h at room temperature in a vacuum oven at 15 lb. pressure. The procedure for GMA embedding, noted in Appendix I, is that of Feder and O'Brien (1968), as modified by Dr. I. Morrison (personal communication).

Sections 1.0 to 6.0 microns thick were cut using glass knives and Sorvall JB-4 microtome. Stains used in addition to acid fuchsin/ toluidine blue (Feder and O'Brien, 1968) included Johansen's Quadruple, Pianeze IIIB, Magdala Red/Fast Green, Thionin/Orange G (all Gurr, 1965; 1971), 1% toluidine blue in 1% sodium borate (modified from Bennett and Radmiska, 1966), trypan blue, and methylene blue (1% in 1% sodium borate) and combinations thereof. Stained sections were examined using a LEITZ ORTHOLUX II microscope and photographed on KODAK Ektachrome 160 tungsten corrected film.

Sections of infected material were treated with fluorochromes ethidium bromide, Calcofluor M2R New, aniline blue, coriphosphene O, or primuline. Free-hand or plastic-embedded sections were treated for

1 min and then washed for 3 min in deionized water. (The free-hand sections had been previously fixed for whole leaf staining in lacto-phenol/alcohol). Both types of sections were examined for fluorescent hyphae using techniques described previously (fluorescent microscopy techniques). Specific applications are noted in Appendix II.

3. Ultra-thin sections

Ultra-thin sections of healthy aerial and floating leaves were processed for electron microscopy as described by Harder et al. (1978). Details of the five schedules used are given in Appendix III. Sections cut with a diamond knife were stained for 10 min in 5% uranyl acetate in 50% ethanol and 10 min in lead citrate. Hitachi HU-12 and AEI EM6B electron microscopes were used to examine the sections.

RESULTS AND DISCUSSION

A. PATHOLOGICAL TECHNIQUES

1. Wild rice (Zizania aquatica) - the host

After-ripened wild rice seed stock, which germinated satisfactorily over a period of 18 months of storage at 4°C in distilled water in the dark, produced healthy plants with fully extended floating and aerial leaves. Seeds, scraped to break dormancy, gave rise to small plants with thin, short leaves and few seeds, which were considered unsuitable for anatomical or pathogenicity studies. The hand-harvested seed stock had a high germination rate (95-100%) which may have been due to the low (4°C) storage temperatures, relatively uncontaminated storage water surrounding the seeds, and the constant dark regime. Hand-harvesting also ensured that all seed was mature at the time of storage.

Suitable greenhouse growth conditions were established by Gilbert (1974), but attempts to duplicate greenhouse conditions in a growth chamber during the 12 weeks from initial seedling stage to maturity resulted in sterile stunted plants with purple pigmented leaves. It appeared some light factor, essential for normal height, leaf development and seed production, was missing. Mature greenhouse-grown plants with aerial leaves and mature spikelets were unaffected by placement in the growth chamber for pre-disposition prior to inoculation and could be left in the growth chamber Model EF 7 for 1 month or more with no abnormality in leaf pigmentation or reproductive ability.

Floating leaves of plants grown either in the greenhouse or the growth chamber were indistinguishable; both became fully extended with normal pigmentation. However, the growth chamber was preferred because

it afforded greater control of temperature and light conditions necessary for floating leaves which are produced in cooler ambient spring conditions in nature.

2. Helminthosporium oryzae - the pathogen

The abilities of thirteen H. oryzae isolates, originally pathogenic to Manitoba wild rice, to sporulate on six growth substrates are tabulated on Table 1. The most vigorously sporulating isolate on all substrates except SSL was FA 72 D#5. Because both filter paper and agar were used separately and in conjunction with autoclaved leaf pieces, the number of spores per plate (Table 1) were counted, although on plates containing leaf segments sporulation was more concentrated on or near these segments than on the surrounding filter paper or agar. In addition, AAL-induced sporulation was generally twice that of AFL-induced sporulation, which may indicate differences in pathogen behavior towards the two leaf types; the pathogen may be responding to differences in the chemical composition of the two leaf types. Lastly, filter paper was more successful than agar as a substrate supplement on AAL and AFL plates.

H. oryzae isolates pathogenic to wild rice and those pathogenic to O. sativa appear to require similar growth conditions for optimum sporulation, such as 27-28°C optimum temperature for conidial formation (Gilbert, 1974; Hau and Rush, 1980), and alternating light/dark photoperiod (Chang, 1974a; Sharma and Singh, 1975; Hau and Rush, 1980). Growth substrates containing plant parts produced abundant sporulation of H. oryzae, possibly accounting for the success of AAL in this study, however, other natural and synthetic media used to induce sporulation have produced minimal results (Sharma and Singh, 1975; Chang, 1980;

Table 1. Induced sporulation of isolates of Helminthosporium oryzae on six growth substrates.*

Fungal isolates	Sporulation of H. oryzae**					
	AAL	AFL	AALSA	AFLSA	SA	SSL
FA 72 D#2	220	140	10	7	x	x
FA 72 D#4	270	180	12	8	x	x
FA 72 D#4A	260	120	11	3	x	x
FA 72 D#5	880	460	82	80	4	1
P#7	34	20	x	x	x	x
BH WP72 D#2C	80	60	3	x	x	x
BH WP72 D#2	90	50	7	x	x	x
K34 GK	520	220	44	36	1	x
K71	130	80	3	x	x	x
K81	180	60	3	x	x	x
LIL 72 D#2	280	100	11	2	x	x
LIL 72 D#2A	620	340	63	51	2	2
LIL 72 D#3	280	90	14	x	x	x

Light treatment: 12 h photoperiod
Temperature: 25°C
Time 14 days
**Conidia x 10^3/plate
x = less than 1000

*Substrates: AAL - autoclaved aerial leaf
AFL - autoclaved floating leaf
AALSA - AAL in Sach's Agar
AFLSA - AFL in Sach's Agar
SA - Sach's Agar
SSL - Sach's Solution on filter paper

Hau and Rush, (1980).

3. Inoculation procedures

On aerial leaves the first conspicuous symptoms were tiny brown lesions; but later three lesion types were distinguishable: (1) lesions 1 mm or less in diameter, dark brown in color referred to as 'discrete' lesions; (2) lesions with a brown centre, surrounded by a chlorotic yellow halo ('haloed' lesions); and (3) large brown spreading lesions that were separated by green tissue. The last were designated as 'coalesced' lesions since they seemed to develop by the coalescence of smaller lesions. Discrete and haloed lesions appeared to retain a definite shape and size and discrete lesions sometimes appeared to be limited by leaf veins. These lesions are illustrated in Fig. 1.

Floating leaves had two types of discrete lesions: (1) those which were similar to aerial leaf 'discrete' lesions; and (2) those with a yellow periphery designated as 'haloed discrete'. Floating leaf lesions with a brown peripheral ring and a yellow centre, measuring approximately 1 mm in diameter, were referred to as 'brown-ringed' lesions. 'Coalesced' lesions similar to those of aerial leaves were also visible. These lesion types are illustrated in Fig. 2.

Although no studies have been reported for floating leaves, these results agree with observations made by Gilbert (1974) and Kernkamp et al. (1976) on infected aerial leaves of wild rice. Typically, on Oryza sativa, H. oryzae caused spots which were oval, relatively uniform and evenly distributed over the leaf surface. They were brown, with grey or whitish centres when fully developed, but young or under-developed spots were small and circular, and appeared as dark brown or purplish brown dots. On susceptible varieties, the spots were much

Fig. 1. Lesion patterns and types on wild rice aerial leaves infected by Helminthosporium oryzae.

Fig. 2. Lesion patterns and types on wild rice floating leaves infected by Helminthosporium oryzae.

A) Floating leaf control.

B,C) Lesions produced by spore concentration #1 at 48 hours post-inoculation.

D,E) Lesions produced by spore concentration #2 at 48 hours post-inoculation.

F,G) Leaves inoculated with spore concentration #2 at 10 days post-inoculation.

H) Infected floating leaves were senescent just prior to sporulation.

Scale = mm


METRIC
1
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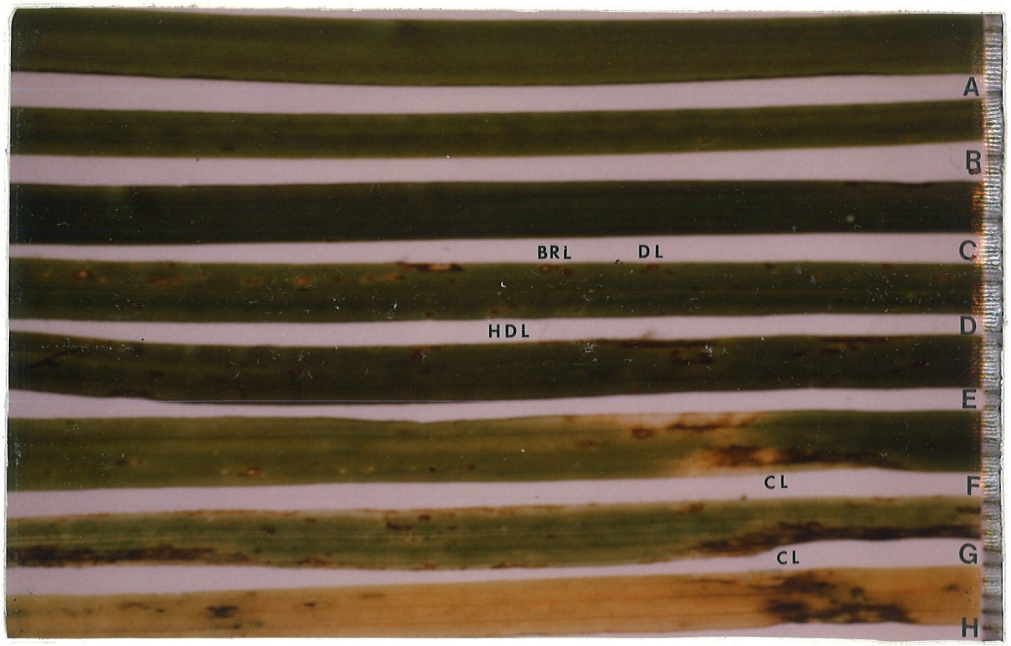
10
CL
CL
HL
CL
DL
DL


A
B
BRL
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E
CL
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CL
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H

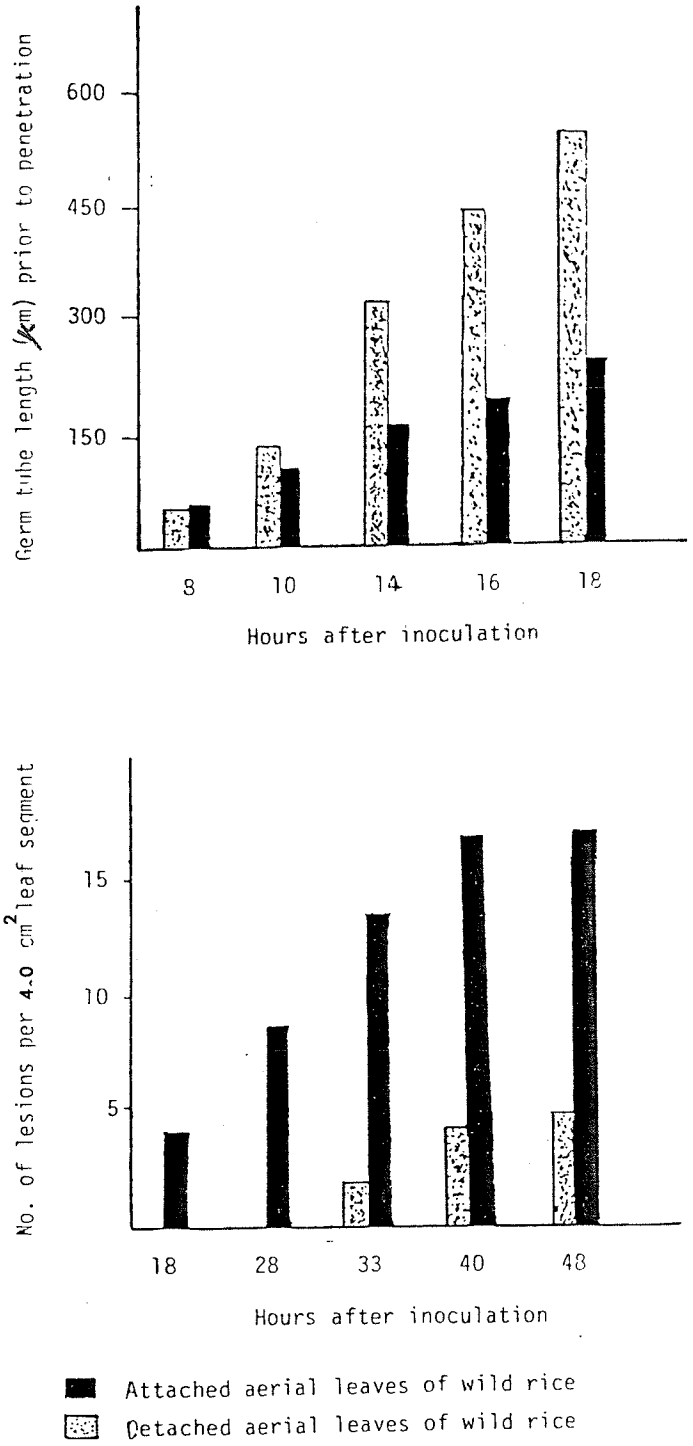
larger and often reached 1 cm or more in length (Ou, 1972). The non-uniformity of the lesions on wild rice may be a consequence of the genotypes of the host and of the pathogen, both of which are extremely heterogeneous (Kernkamp et al., 1976).

The development of lesions on wild rice plants inoculated with isolate FA 72 D#5 confirmed its virulence. Subsequent experiments were carried out to determine if detached aerial leaves could be used for infection studies. "Detached" leaves, which had been removed and cut into 4.0 cm^2 segments prior to inoculation, were compared with "attached" leaves, remaining on the plant throughout the inoculation and incubation procedures. The results are shown in Figs. 3 and 4. On detached leaves, H. oryzae developed superficially up to about 35 h post-inoculation (p.i.) before lesions appeared, while on attached leaves, less hyphal growth occurred prior to penetration and initial lesion formation began at 18 h p.i. These differences may be attributable to the saprophytic capability of the fungus which given a host beginning to undergo senescence (as indicated by loss of chlorophyll), will colonize with superficial mycelia. Unfortunately the benzimidazole treatment delayed, but did not prevent senescence. As a result, only attached aerial leaves were used in subsequent infection studies.

Since an inoculum dosage of 1.25×10^6 spores per 25 plants did not give sufficient lesions per floating leaf for convenient microscopic examination (Fig. 2, B, C), the volume of inoculum was doubled. This resulted in a large number of the four lesion types being produced (Fig. 2, D-G). Under natural conditions floating leaves usually produce only a few lesions; nevertheless, it appears that when the inoculum concentration was high, floating leaves were quite susceptible

Fig. 3. Germ tube length (μm) prior to penetration on attached or detached wild rice aerial leaves vs. Hours after inoculation.

Fig. 4. Number of lesions per leaf segment (4.0 cm^2) on attached or detached wild rice aerial leaves vs. Hours after inoculation.


Germ tube length (μm) prior to penetration
600
450
300
150
8
10
14
16
18
Hours after inoculation
No. of lesions per 4.0 cm² leaf segment
15
10
5
18
28
33
40
48
Hours after inoculation
Attached aerial leaves of wild rice
Detached aerial leaves of wild rice

to attack, even at lower ambient temperatures than aerial leaves. Just prior to sporulation floating leaves were senescent, and the lesion types showed distinctly against the host background (Fig. 2, H).

The conditions used for the successful inoculation of aerial leaves were ascertained by Gilbert (1974). Those for floating leaf inoculations were chosen to emulate the cooler conditions encountered by emerging floating leaves in the spring. Kernkamp et al. (1976) noted that blight increased on wild rice when daytime temperatures were 30-35°C and RH ranged from 70-90% at night plus dew. They recommended inoculation conditions of 22°C for 48 h in a moist chamber and then return to 24°C in the greenhouse. Sherf et al. (1947) found that the presence of free water on the leaves of O. sativa at the time of inoculation appeared to favor infection, but that a hard rain immediately following inoculation of H. oryzae washed the conidia from the leaves before penetration. They also found that evening inoculations gave more consistently severe infection, that the optimum temperature range was 20-25°C, and that conidia were five to ten times more effective than hyphal fragments, however Lam and Thrower (1973) found that hyphal fragments of H. oryzae infected rice plants in a manner comparable to conidia. Disease development was found to be optimum at 25-30°C and 90-100% RH (Sarkar and Sen Gupta, 1977). Purkayastha and Mukhopadhyay (1974) suggested that spore concentrations of 4.0×10^4/mL or 2.0×10^4/mL water from 10-30 day old cultures gave satisfactory infection of rice and that leaf exudates stimulated germ tube growth significantly.

It appears, then, that wild rice isolates of H. oryzae require similar conditions for infection to those from rice. This may help to explain the more frequent epidemic outbreaks of H. oryzae blight in the

southern portion of the distribution range of wild rice.

B. HOST-PATHOGEN INTERACTION #1: Wild rice aerial leaves/ *H. oryzae*

1. Histology of *Zizania aquatica* - the healthy host

a. Gross anatomy

The anatomical features of the aerial leaves are illustrated in Fig. 5.

Both the adaxial and abaxial epidermis consisted of alternating costal and intercostal zones (Fig. 5, A-D). The costal zone was characterised by silica cells and cork cells. The silica cells (Fig. 5, B) which were predominantly over the veins contained silica bodies of the *Oryza*-type and were separated by cork cells. Silica cells were also found in the stomatal band. Also in the intercostal zone of the adaxial surface were zones of bulliform cells adjacent to rows of long cells, and a stomatal band consisting of stomates and interstomatal cells on either side of a vein. The abaxial surface was similar, but lacked bulliform cells in the intercostal zone. In surface view, it was possible to observe that stomates had dumb-bell shaped guard cells, roughly triangular subsidiary cells, and adjacent interstomatal cells with concave walls. Four papillae overarched each stoma. Both short and long cell walls were wavy in outline, while bulliform cell walls were not. Papillae occurred on the outer walls of most cells.

In transverse section, a well-defined mid-rib projected from the abaxial surface (Fig. 5, E). This had large lacunae or air spaces which were separated from each other by thin radiating bands of parenchyma. The lacunae were septate and their transverse diaphragms were composed of stellate cells, which supported vascular tissue. The cellular detail of the mid-rib is illustrated in Fig. 5, F. The parenchyma cells were thin-walled and lacked chloroplasts, however chlorenchyma cells were

Fig. 5. The anatomy of wild rice aerial leaves.

Adaxial surface (A-C)

A) Pattern of alternating rows of epidermal cells (AB;F2-I). x 117.

B) Bulliform cells, stomates, silica bodies, long cells, and interstomatal cells on either side of a vein (AB;F2-II). x 466.

C) Nuclei of cells, and walls of long cells (EB;F3-II). x 466.

Adaxial surface (D)

D) Stomates, silica bodies and other epidermal cells; bulliform cells lacking (AB;F2-II). x 400.

Transverse sections (E-L)

E) Hand section of Zizania-type mid-rib showing lacunae, transverse diaphragm; cellular detail in mesophyll area lacking (C;F1-I). x 117.

F) Cellular detail of Zizania-type mid-rib (1) thin-walled parenchyma cells surrounding lacunae and chloroplast-containing cells underlying the epidermis; (2) sclerenchyma; (3) vascular bundle and bundle sheath (AF/TB;-). x 154.

G) Large vascular bundle with outer bundle sheath cells containing chloroplasts, thick-walled inner bundle sheath cells, xylem and phloem (AF/TB;-). x 508.

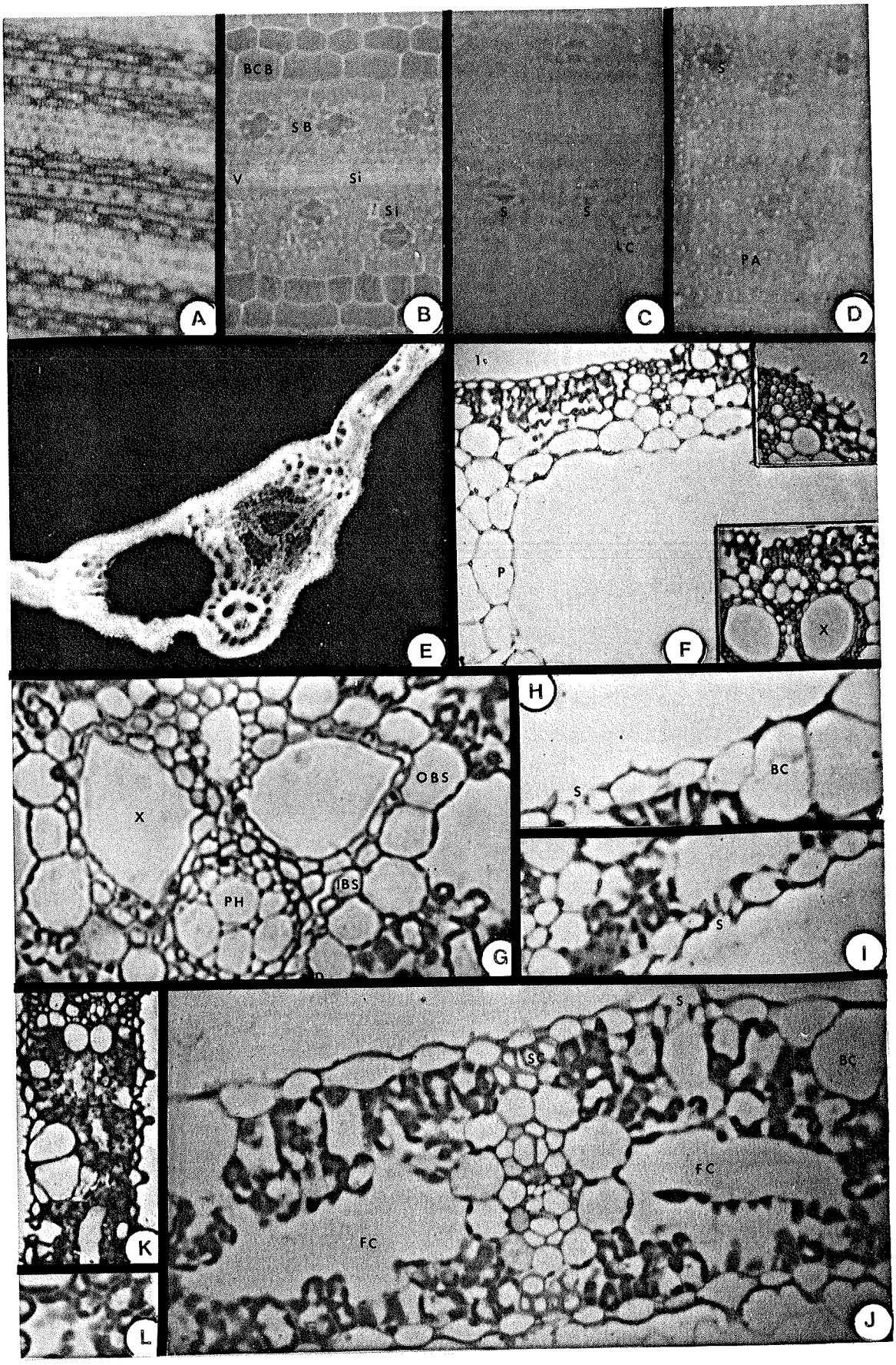
H) Adaxial epidermis showing guard cells, subsidiary cells, thick-walled cells adjacent to the stomate, and bulliform cells with differentially stained outer walls (AF/TB;-). x 508.

I) Abaxial epidermis showing stomate, substomatal chamber. Thick-walled cells adjacent to the stomate have papillae (AF/TB;-). x 508.

J) Lamina showing fusoid cells adjacent to a small vascular bundle, double bundle sheath, arrangment of mesophyll cells (arm-type parenchyma), sclerenchyma, and silica cells associated with the vascular bundle (AF/TB;-). x 508.

K) Lamina showing cuticle, epidermal cell types, bundle sheath, and arm-type parenchyma cells (MB;-). x 266.

L) Arm-type parenchyma cells with chloroplasts (TB;-). x 508.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observed underlying the epidermal cells. The epidermis was composed of short and long cells. Sclerenchyma cells and vascular bundles were arranged in a complex pattern. Bulliform cells occurred at the junction of the mid-rib and the lamina.

The constitutent cells of the lamina proper are shown in transverse section in Fig. 5, G-L. The cells associated with the vascular bundles were similar in both the mid-rib and the lamina. All vascular bundles had two complete bundle sheaths (Fig. 5, G); the outer consisted of large, thin-walled cells with parietal chloroplasts; the inner of smaller thick-walled cells. The large metaxylem vessels gave an angular shape to the vascular bundles. The vascular bundles were subtended both above and below by sclerenchyma cells; the larger the vascular bundle, the more numerous were the sclerenchyma cells. The transverse section of the epidermis revealed differences in cell walls and papillae between the constituent cells. The stomatal complex consisted of chlorophyll-bearing guard cells, associated with subsidiary cells and overarching papillae (Fig. 5, H). Bulliform cell walls appeared to stain differently from those of adjacent short and long cells. Cell wall thickness and staining of abaxial epidermal cells (Fig., 5 I) were generally similar to those of the adaxial epidermis. All epidermal cells appeared to be highly vacuolated and lacking in cytoplasm and organelles. The chlorenchyma was uniformly characterized by cell wall invaginations (Fig. 5, J-L), a pattern which Metcalfe (1960) termed 'arm cells' or 'arm-type parenchyma cells'. Fusoid cells, often incorrectly interpreted as intercellular spaces (Metcalfe, 1960) were present in wild rice on either side of all but the smallest vascular bundles (Fig. 5, J).

These observations broadly conform with earlier reports on the

anatomy of Zizania spp. Weir and Dale (1960) noted similar epidermal and mid-rib features in Z. aquatica var. angustifolia, however, no cellular descriptions were presented. Using scanning electron microscopy, Terrell and Robinson (1974) and Terrell and Wergin (1979) observed arm cells, Oryza-type silica bodies, a complex system of vascular bundles in the mid-rib, air chambers, fusoid cells, and overarching stomatal papillae in Z. aquatica varieties and Z. latifolia. It is not surprising that Oryza sativa and Leersia spp. (also members of Oryzeae) have many characteristics of leaf morphology in common with wild rice (Holm, 1892, 1895, 1896; Tullis, 1935, Kaufman, 1959; Metcalfe, 1960; Terrell and Robinson, 1974; Shekhawat and Patel, 1978; and Hau and Rush, 1982), however both these genera lacked fusoid cells. The similarity of leaf morphology in Zizania, Oryza, and Leersia is important to plant pathologists since isolates of Helminthosporium oryzae have been found to be pathogenic to members of all three genera. Chang (1974b; 1975) found that three isolates from Z. aquatica were pathogenic to both O. sativa and Z. latifolia. H. oryzae was also found to attack Leersia hexandra, under natural conditions (Chattopadhyay and Chakrabarti, 1953). In all probability the host range of H. oryzae is related to the leaf morphology of potential hosts, that of Oryzeae being preferred.

b. Evaluation of histological techniques

i. Fluorochromes

Cleared whole mounts of leaf segments and plastic-embedded leaf sections treated with fluorochromes were used to study aerial leaf morphology. For cleared leaves, aniline blue (AB) and ethidium bromide (EB) in conjunction with epifluorescence microscopy gave excellent results.

AB stained cell walls and highlighted their associated papillae (Fig. 5, A,B,D). Oryza-type silica bodies fluoresced brightly in both the stomatal band and over the veins. Cell contents remained relatively unstained. These results support Smith and McCully's (1978a) contention that AB will bind to most wall structures, that it did not specifically indicate the presence of β-1,3-glucan, as earlier suggested (Eschrich and Currier, 1964), and that the same structure was often both autofluorescent and AB fluorescent. Other authors (Currier and Strugger, 1956; Dionne and Spicer, 1958; Martin, 1959; Faulkner et al., 1973, Jeffries and Belcher, 1974; Smith and McCully, 1978b; Polito and Luza, 1981) have reported similar findings using free-hand as well as paraffin or glycol methacrylate embedded sections of plant material.

EB imparted a bright orange-red fluorescence to all nuclei and cell walls and emphasized the position of the four overarching papillae associated with the stomata (Fig. 5, C). EB has been shown to bind to DNA and RNA (Elliott, 1963; LePecq and Paoletti, 1966; 1967; LePecq, 1971; Burns, 1972). Roser (1980) reported that in wheat root cells stained with EB, both the nuclei and cell walls were stained, and exhibited a characteristic orange-red fluorescence. O'Brien and McCully (1981) noted that EB had been used to stain xylem. The results of this study further indicate that EB reacts with plant cell walls of many types.

The optical brightener, Calcofluor, a fluorochrome of the diaminostilbene-disulphonate type, was used to treat hand sections of leaf material previously cleared with lactophenol. Calcofluor preferentially stained the walls of highly vacuolate cells such as bulliform cells and parenchyma cells associated with the vascular bundle (Fig. 5, E).

Mesophyll cells exhibited a characteristic golden-yellow autofluorescence which overwhelmed the Calcofluor-induced blue fluorescence. Plastic-embedded sections, treated with Calcofluor, yielded similar results. Hughes and McCully (1975), Peterson et al. (1978) and Peterson and Peterson (1980) also found that when Calcofluor was used to treat cells of higher plants in either free-hand or plastic-embedded sections, the cell walls were brilliantly blue fluorescent, but normally the cytoplasmic components were unstained. This pattern is due to the ability of Calcofluor to bind to cellulose, chitin, and other β-linked polymers (Maeda and Ishida, 1967; Harrington and Raper, 1968; Hayashibe and Katohda, 1973).

The use of fluorochromes with healthy wild rice tissue is summarized in Appendix II. Fluorochromes which did not give adequate differentiation were Rhodamine B, Auramine O, acriflavine, and picridine orange. Coriphosphene O, Nile Blue A and primuline did cause cell walls to fluoresce, however the differentiation was not as great as that of either AB, EB or Calcofluor and the results were unsatisfactory for photography. Congo Red was found to stain veins on cleared leaves in a manner similar to AB, but as it was not superior such preparations were not photographed.

ii. Conventional stains

JB-4 plastic-embedded leaf sections stained with acid fuchsin/toluidine blue (AF/TB) (Feder and O'Brien, 1968) clearly differentiated between the epidermis and mesophyll (Fig. 5, F-J). The outer walls of the bulliform cells had a purplish hue in contrast to the bright blue of the other abaxial and adaxial epidermal cells. Chloroplasts of the mesophyll cells and the thin-walled cells of the outer bundle sheath

stained purple. The thick walls of the inner bundle sheath cells were light blue, while most lignified walls were bluish-green. Minor differences in shade and tint from those reported by Feder and O'Brien (1968) were probably due to the use of a different type of glycol methacrylate plastic.

Methylene blue (MB) (1% in 1% soidum borate) differentiated arm-type parenchyma (or chloroenchyma) cell walls, chloroplasts and nuclei (Fig. 5, K), and its use confirmed the observations made using other stains. However, it was not able to differentiate epidermal cell walls as well as AF/TB or TB alone. TB offered no advantage over MB as a stain for differentiating mesophyll cells (Fig. 5, K,L). Other conventional stains tested were less effective than AF/TB and MB and the results were unsatisfactory for photography.

Bulliform cell walls appeared to stain differently from those of adjacent short and long cells (Fig. 5, H). This may be due to the fact that bulliform cell outer walls remain in a pectinocellulosic state long after other cells have become lignified (Whitney, 1976). Appressoria of H. oryzae form preferentially over bulliform cells on O. sativa leaves and this may be related to the nature of the cell wall (Hau and Rush, 1982).

iii. Electron microscopy procedures

Wild rice leaf tissue is delicate and refractory due to the large amount of air space present within the leaf and the high degree of vacuolation in many of the cells. Thus techniques which were specifically developed for sensitive plant tissues (Fowke, 1975; Mills and Chong, 1977; Harder et al., 1978) were progressively modified until satisfactory

results were obtained. The specific schedules of five procedures are given in Appendix III. The results of procedures 1, 2 and 5 are presented in Fig. 6.

Procedure 1, a modification of the technique used for rapeseed by Mills and Chong (1977), incorporated the same fixation procedures as were used successfully to produce plastic-embedded thin sections of wild rice tissue. As Spurr's resin (Spurr, 1969) is miscible with ethanol in all proportions, no transitional solvent was used. The shrunken and disorganized appearance of aerial leaf mesophyll cells caused by poor infiltration is illustrated in Fig. 6, A. The plasmalemma and chloroplast membranes lacked integrity. Components of nuclei and mitochondria were not discernible and the cytoplasmic ground substance showed empty spaces.

The poor infiltration of the Spurr's resin was alleviated somewhat by incorporating propylene oxide as a transitional solvent between ethanol and Spurr's resin (procedure 2). Propylene oxide has often been used to eliminate the problem of non-uniform impregnation of sensitive tissue (Hayat, 1970; Fowke, 1975). Although overall cellular definition was improved and delimiting organelle membranes and cytoplasmic ground substance were better defined, membrane clarity was not improved (Fig. 6, B). In addition, a great deal of precipitate was present in the chloroplasts and mitochondria. Dr. J. Chong (personal communication) indicated that similar precipitates had been observed in wheat leaf cells because of short washing time between glutaraldehyde and osmium tetroxide fixation. He also suggested that the membrane distortions could be due to acrolein, which might increase the extractable lipid (Hayat, 1970).

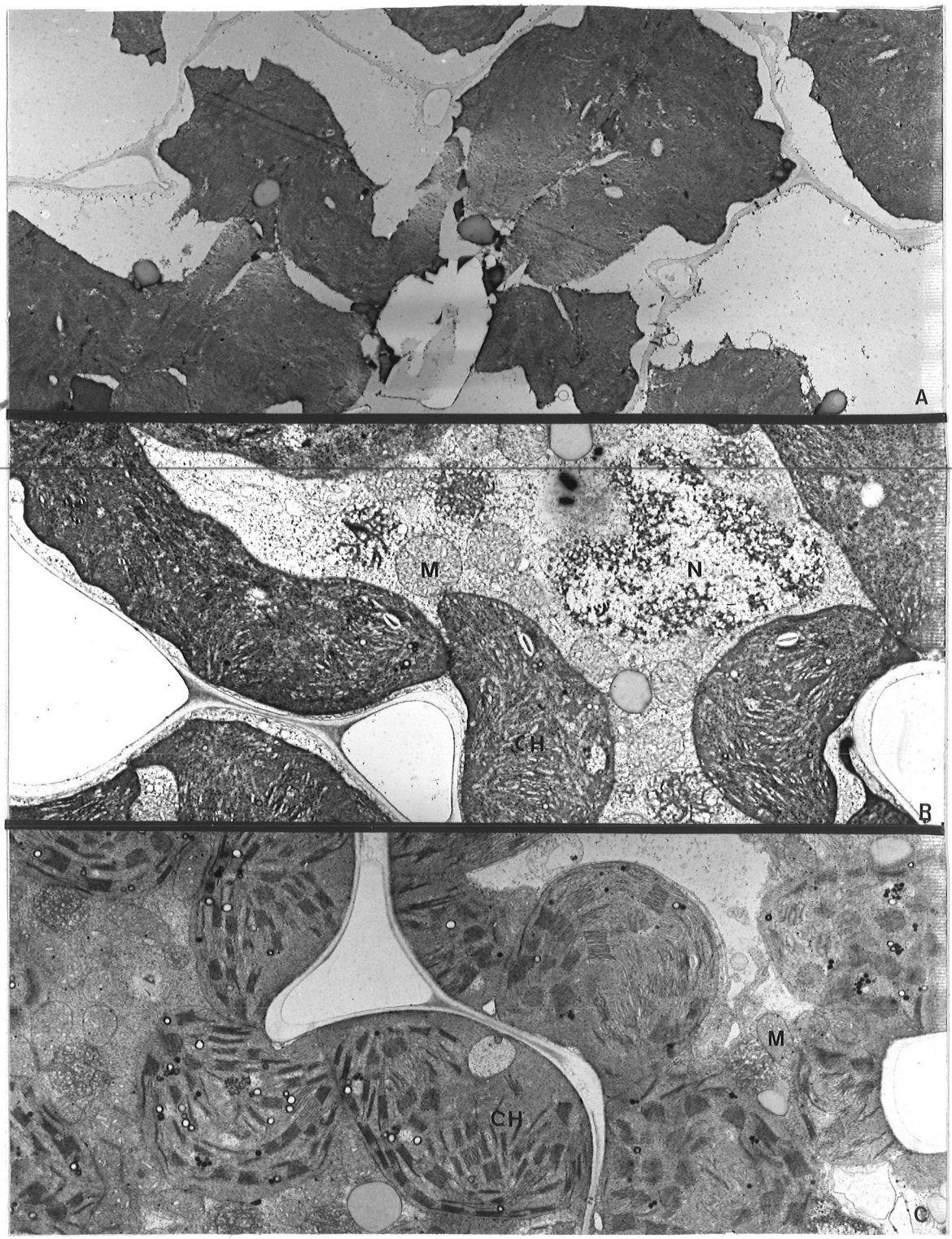
Fig. 6. A comparison of procedures used to prepare wild rice aerial leaves for electron microscopy.

Mesophyll cells

A) Procedure #1. Organelles show a complete lack of detail. x 7000.

B) Procedure #2. Organelle structures are visible, but ill-defined; chloroplasts appear distorted with no clear thylakoid organization. x 10 000.

C) Procedure #5. Organelles are clearly defined; chloroplasts show rounded outline and well-defined thylakoids. x 8000.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Procedure 3, then, involved the removal of acrolein from the fixative, and the addition of a much longer washing time in phosphate buffer and post-fixation with osmium tetroxide, followed by a more gradual dehydration in ethanol. Fixation was accomplished under partial vacuum to ensure rapid fixation and better access of the tissue to the chemicals in the later stages. As a result, the precipitate in the organelles was notably less; and the membranes of the organelles appeared to be more defined, although they were not completely intact. Procedure 4, incorporating the modified fixation and dehydration steps of procedure 3 with the propylene oxide-free infiltration of Spurr's resin unfortunately resulted in distortion similar to that of Fig. 6, A, thus pointing to the dehydration stage as the cause of the residual distortions.

Procedure 5 combined the procedure 3 fixation technique with the gradual ethanol-propylene oxide dehydration (transition) series. The elimination of acrolein, the use of partial vacuum, a longer washing time with phosphate buffer and post-fixation in osmium tetroxide, followed by the more gradual dehydration produced satisfactory cellular detail (Fig. 6, C). Mesophyll cell walls showed no breaks and the plasmalemma was clearly delimited and intact. Chloroplast membranes and lamellae were intact and thylakoid definition was excellent. Mitochondria had intact outer double membranes and a dense matrix; cristae were well defined. Lipid bodies were also well defined and rounded. These characteristics fit Hayat's (1970) description of cells which are well-fixed for transmission electron microscopy. Examinations of other cell types of wild rice aerial leaves indicated that procedure 5 should be used routinely for this leaf type.

c. Ultrastructure of healthy cells

Wild rice epidermal cells had thick walls with a layered granular arrangement (Fig. 7). Those adjacent to the stomatal complex were highly vacuolate and more or less devoid of organelles. Subsidiary cells, on the other hand, had abundant cytoplasm while guard cells were well-endowed with organelles - nuclei, mitochondria, and chloroplasts. Cells of the stomatal complex appeared to have thinner walls than the adjacent short cells (Fig. 7, A). The thick outer wall of the epidermal cells had densely staining granules in the outer layers (Fig. 7, B). The papillae present on most epidermal cells were projections of the cell walls and consisted of similar layers (Fig. 7, C). Oryza-type silica bodies (Fig. 5, B,D) were embedded in the concavity of silica cell walls (Fig. 7, D).

Arm-type parenchyma cells were lobed; their chlorplasts were near the cell walls, while nuclei and lipid bodies occupied central positions (Fig. 8, A). Mitochondria were dispersed throughout these cells. Starch granules, although numerous near the nucleus in one cell (Fig. 8, B) were not usually abundant in any of the cells. Lipid bodies, however, were abundant (Fig. 8 C,D) and were found in association with most organelles. Chloroplasts of these cells had a well developed thylakoid structure (Fig. 8, E).

Wild rice bundle sheath cells contained chloroplasts which differed structurally from those of the arm cells (Fig. 9). In addition, parenchyma cells associated with phloem and xylem elements possessed structures which resembled microbodies, defined by Gunning and Steer (1975) as small bodies bounded by a single true membrane and containing a granular to crystalline matrix. Such structures are illustrated in

Fig. 7. Ultrastructure of epidermal cells of wild rice aerial leaves.

A) Epidermis showing the stomatal complex and an adjacent thick-walled epidermal cell. x 10 000.

B) Layering of the thick wall of an epidermal cell. x 40 000.

C) Papilla of an epidermal cell to show continuity of layers with a cell wall. x 16 000.

D) Oryza-type silica body. x 20 000.

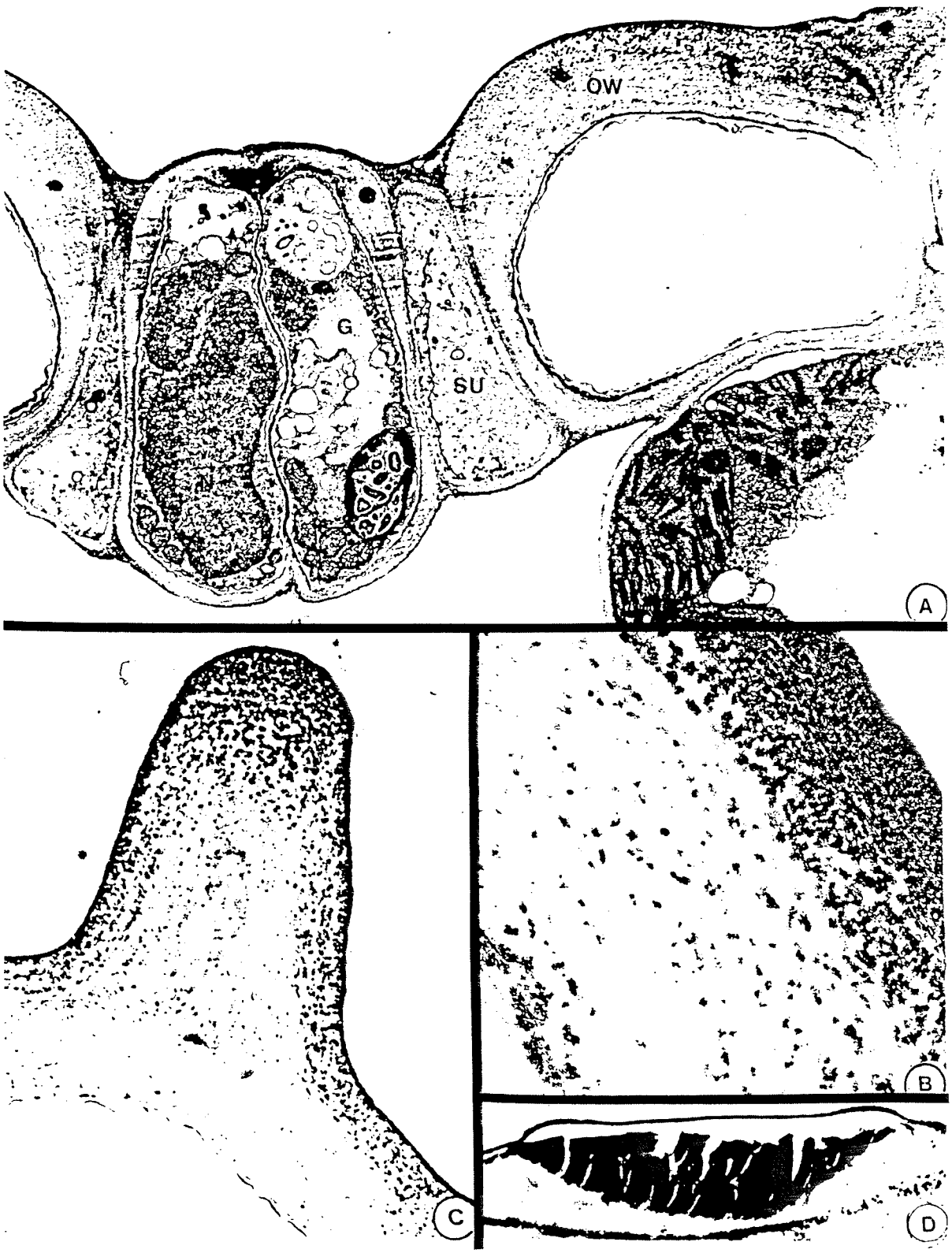
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Fig. 8. Ultrastructure of mesophyll (arm-type parenchyma) cells of wild rice aerial leaves.

A) Overview showing an invaginated wall and the relative positions of the organelles. x 8000.

B) Nucleus surrounded by abundant starch uncommon in mesophyll cells. x 9600.

C) Nucleus with prominent nucleolus and associated lipid bodies common in mesophyll cells. x 25 000.

D) Mitochondria and lipid bodies in association with a chloroplast. x 15 000.

E) Well-defined thylakoid structure and osmiophilic globules within a chloroplast. x 50 000.

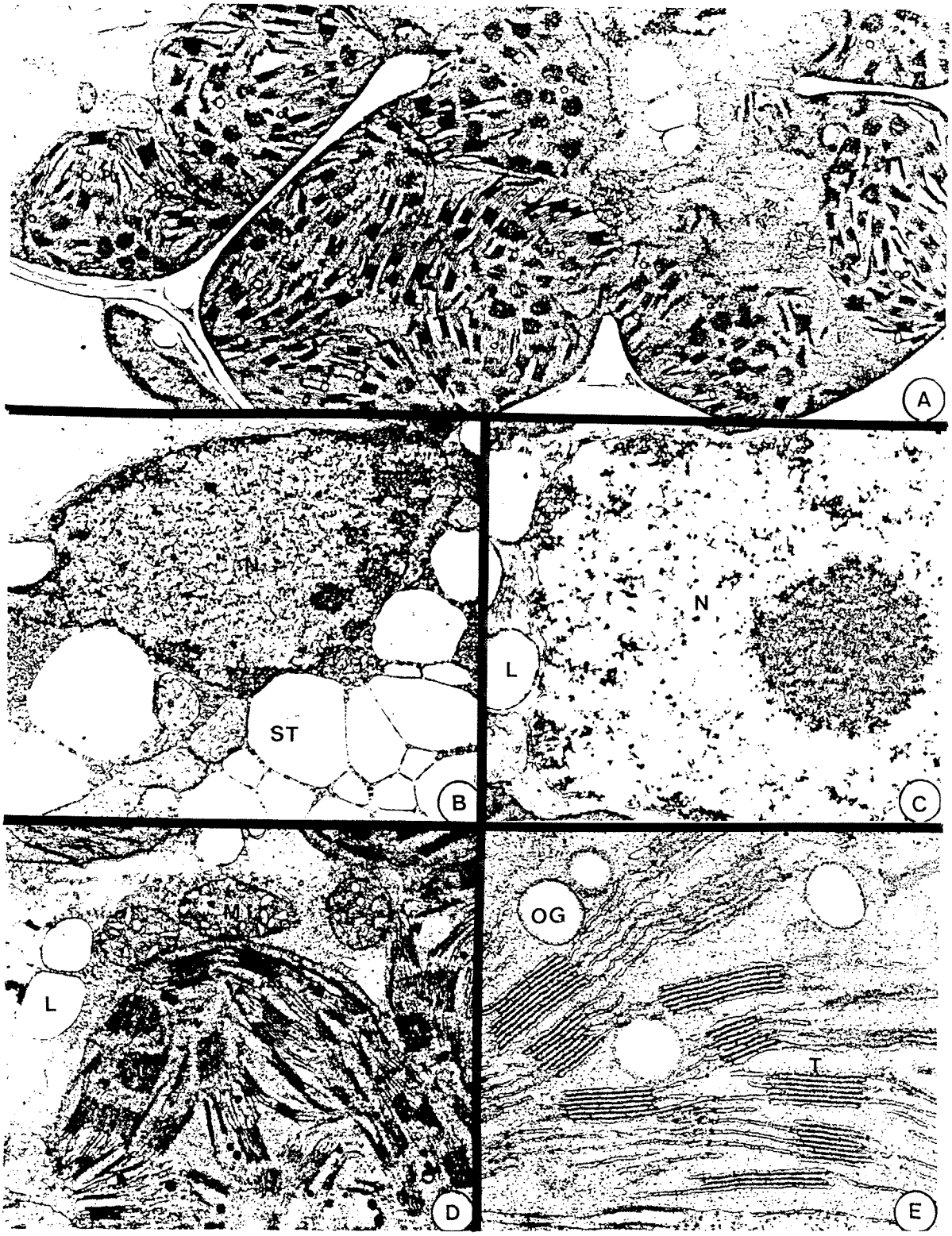
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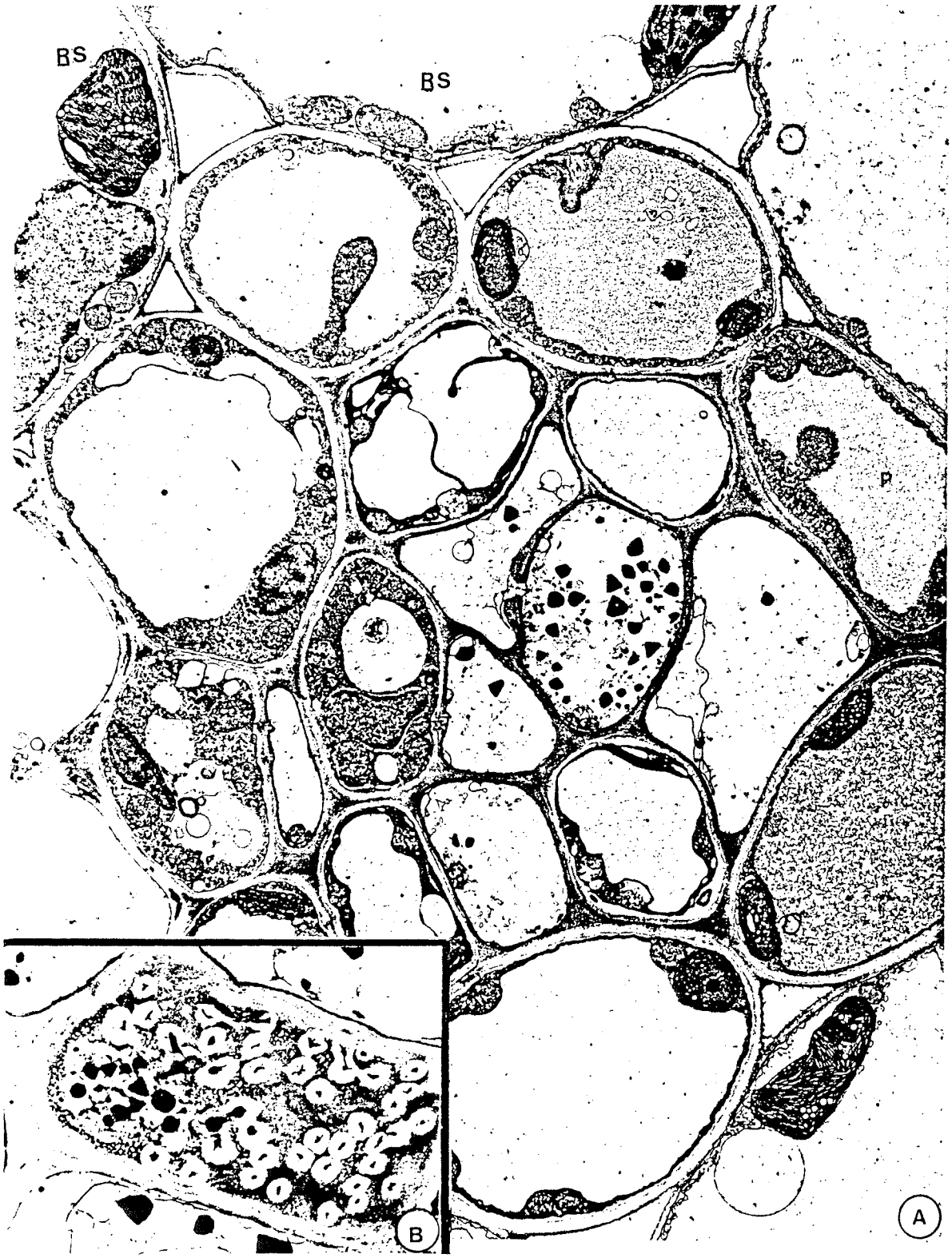
Fig. 9. Ultrastructure of a vascular bundle of wild rice aerial leaf. I. Phloem cells.

A) Sieve elements, surrounding parenchyma cells, and bundle sheath. x 7000.

B) Sieve plate. x 14 400.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Figs. 9 and 10. The crystal (Fig. 10, B) was identified as the enzyme ribulose diphosphate carboxylase (Dr. M. Gibbs, Dr. S. Badour, personal communication). Chloroplasts, often structurally different, in both bundle sheath and mesophyll cells, and RUDP-carboxylase in the bundle sheath, but not the mesophyll cells are characteristic features of C-4 plants, according to Gunning and Steer (1975). However, this circumstantial anatomical evidence for C-4 metabolism in wild rice should be tested by physiological experimentation before firm conclusions are drawn.

It is possible to offer an alternative explanation for these microbody-like structures which are characterized by a curved electron dense rod or plate parallel with their long side (Fig. 10). Berger et al. (1981) have published an electron micrograph of a wild rice mesophyll cell infected by wheat streak mosaic virus (WSMV) in which a rod, strikingly similar in size and shape, is associated with circular and pinwheel inclusions. The rod, in their case, was not within a membrane bound organelle nor associated with crystalline material; nor were there any external symptoms of WSMV infection in the leaf from which Figs. 9 and 10 were prepared. However, the occurrence of these rods in both cases, suggests that the microbody-like structures with their crystalline and circular figures may also be related to the presence of a virus.

Aerial leaf cells of wild rice (Figs. 7-9) are similar in ultrastructure to those of other plants (Jenson, 1970; Ledbetter and Porter, 1970; Gunning and Steer, 1975), and in particular O. sativa (Horino, 1973). However, this study has confirmed that the appearance of organelles and especially chloroplasts (Fig. 6) can be dramatically

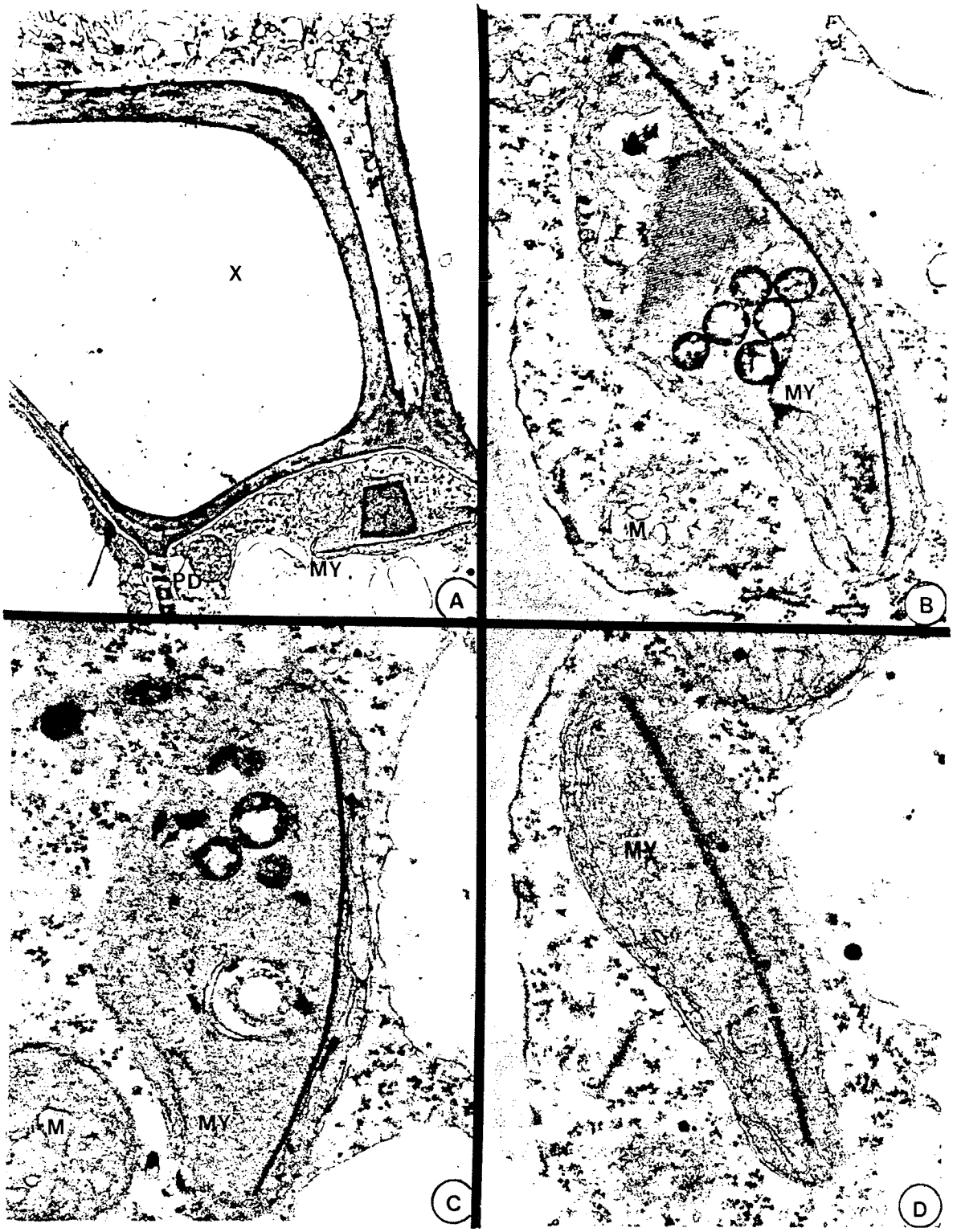
Fig. 10. Ultrastructure of a vascular bundle of wild rice aerial leaf. II. Xylem and microbodies.

A) Walls of vessel elements and parts of adjacent parenchyma cells showing plasmodesmata and 'microbody' containing a crystal and characteristic electron dense rod. x 11 500.

B) 'Microbody' with crystal, characteristic electron dense rod, and circular figures. x 42 500.

C) 'Microbody' with electron dense rod and circular figures. x 42 000.

D) 'Microbody' with electron dense rod. x 42 000.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affected by preparative techniques, as indicated by Weibull et al. (1980). When host-pathogen interactions are being examined, therefore, infected tissues must be rigorously compared with healthy control specimens.

2. The disease cycle

For the purpose of this discussion, the disease cycle was divided into four distinct stages (Agrios, 1969; Jones and Clifford, 1978): (1) pre-penetration (Fig. 11, A-D); (2) penetration (Fig. 11, E-O); (3) lesion formation and development (Figs. 12 and 13); and (4) sporulation of the pathogen on the host.

a. Pre-penetration

The pre-penetration activities of Helminthosporium oryzae isolate FA 72 D#5 on the aerial leaves of wild rice are illustrated in Fig. 11, A-D. Conidia germinated at approximately 2.5 - 4.0 h post-inoculation (p.i.). Germ tube branching and appressorium formation followed germ tube initiation either from one or both ends of the spore. Orientation of the germ tubes appeared to be random and the longer the germ tube, the less likely that it would terminate in an appressorium. At 18 h p.i., most germ tubes were well developed, branched and forming appressoria, which varied in shape from circular to oval to spade-shaped, and ranged widely in size (up to 10μm). These observations were similar to those reported for Helminthosporium oryzae on O. sativa by Locci (1969) and more recently by Hau and Rush (1982).

b. Penetration

H. oryzae penetrated wild rice aerial leaves through stomates, anticlinal walls of interstomatal cells, and wall junctions of bulliform cells (Fig. 11, E-O). Penetration was usually preceded by the formation of an appressorium, although in the case of stomates, appressoria were not always present. The first indication of successful penetration appeared to be a bright yellow autofluorescence of the walls or contents of affected host cells. Stomatal penetrations

Fig. 11. Initial stages of infection of wild rice aerial leaves by Helminthosporium oryzae.

A) Orange-red fluorescent germ tube of the pathogen showing branches terminating in appressoria at 18 h p.i. (EB;F3-II). x 333.

B) Reddish fluorescence of fungal nuclei and germ tube showing an alternate germination pattern at 18 h p.i. (EBs;F3-II). x 366.

C) Blue fluorescence of germ tubes from spore showing bipolar germination at 18 h p.i. (C;Fl-I). x 533.

D) Circular appressorium over a stoma at 18 h p.i.; no visible symptoms (C;F3-I). x 466.

E) Stomatal penetration showing a blue-fluorescent spade-shaped appressorium and yellow autofluorescence of host cells at 22 h p.i. (C;Fl-I). x 333.

F) As E), but with Calcofluor-fluorescence quenched to accentuate autofluorescence of guard cells and underlying mesophyll cells (C;Fl-I). x 333.

G) Effect of stomatal penetration on stomatal complex and underlying mesophyll cells at 24 h p.i. Note unstained tips of over-arching stomatal papillae (MB;-). x 1380.

H) Infected interstomatal cells sometimes contain hyphae and show thickened cell walls at 26 h p.i. (MB;-). x 1380.

I) Silica cells, cork cells and sclerenchyma cells associated with vascular bundles show thickened walls and intracellular hyphae at 26 h p.i. (MB;-). x 1380.

J) Germ tube with oval appressorium situated over a bulliform cell at 26 h p.i. (C;Fl-I). x 333.

K) As J), but with Calcofluor-fluorescence quenched to accentuate autofluorescent bulliform cell walls adjacent to the penetration point (C;Fl-II). x 333.

L) Multiple penetration of bulliform cell walls by branches of one germ tube at 30 h p.i. is extremely rare (C;Fl-I). x 400.

M) As L), but with Calcofluor-fluorescence quenched to accentuate yellow autofluorescence which indicates affected bulliform cells (C;Fl-II). x 400.

N) Direct penetration of a bulliform cell by a germ tube from an H. oryzae conidium showing granular host cytoplasm and hyphal entry into an adjacent epidermal cell (MB;-). x 1380.

O) Intercellular invasion of mesophyll by a hyphae at 33 h p.i. following epidermal penetration (MB;-). x 1380.

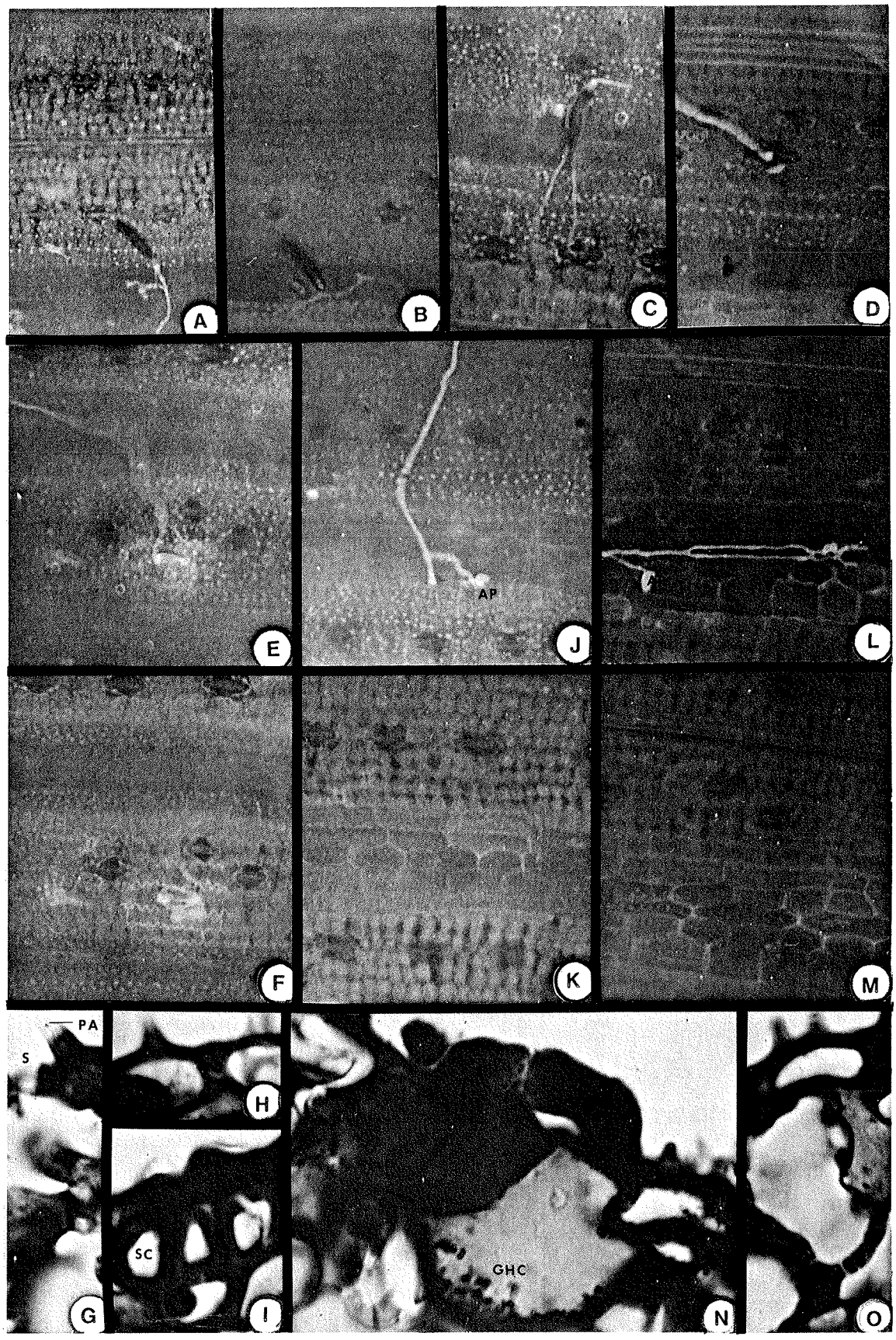
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Fig. 12. Lesion development on wild rice aerial leaves infected by Helminthosporium oryzae.

33 to 48 hours post-inoculation (A-J)

A) Small brightly autofluorescent lesion in the stomatal band (C;Fl-I). x 466.

B) Bright yellow autofluorescence of three affected mesophyll cells underlying a penetration point in the interstomal region (C;Fl-II). x 466.

C) Circular expansion of autofluorescent area as lesion size increases (C;Fl-I). x 466.

D) Number of autofluorescent mesophyll cells increases as lesion develops (C;Fl-II). x 466.

E) Intense autofluorescence of epidermal cell walls and underlying mesophyll cells (C;Fl-II). x 466.

F) Formation of a dark-pigmented band of cells surrounding central autofluorescent cells (C;Fl-I). x 466.

G) Lesion showing three zones of host reaction: (1) cells of the initial infection site remain autofluorescent; (2) surrounding cells are dark brown and non-fluorescent; (3) an adjacent zone of cells are also non-fluorescent and grey-brown (C;Fl-I). x 466.

H) As G), but Calcofluor-fluorescence quenched to accentuate host response (C;Fl-II). x 466.

I) Lesion formation in the stomatal band showing initially infected autofluorescent cells and brown pigmentation of the surrounding cells spreading to the vascular area (C;Fl-I). x 466.

J) Lesion showing reddish brown pigmentation in bulliform cells adjacent to an initial stomatal band infection (C;Fl-II). x 400.

7 days post-inoculation (K-O)

K) Dark brown pigmented discrete lesion with associated appressorium and no autofluorescent areas (C;F3-I). x 200.

L) Transverse section through a discrete lesion showing the limited extent of host infection (MB;-). x 154.

M) Well-developed lesion in which initially infected host cells remain autofluorescent while later affected cells have become deeply brown pigmented (C;Fl-I). x 200.

N) Transverse section through a coalesced lesion showing extensive collapse of mesophyll and vascular bundle with resultant shrinkage of the leaf (MB;-). x 154.

O) Transverse section through a haloed lesion. Note greater collapse at the centre of the lesion than at the periphery (MB;-). x 154.

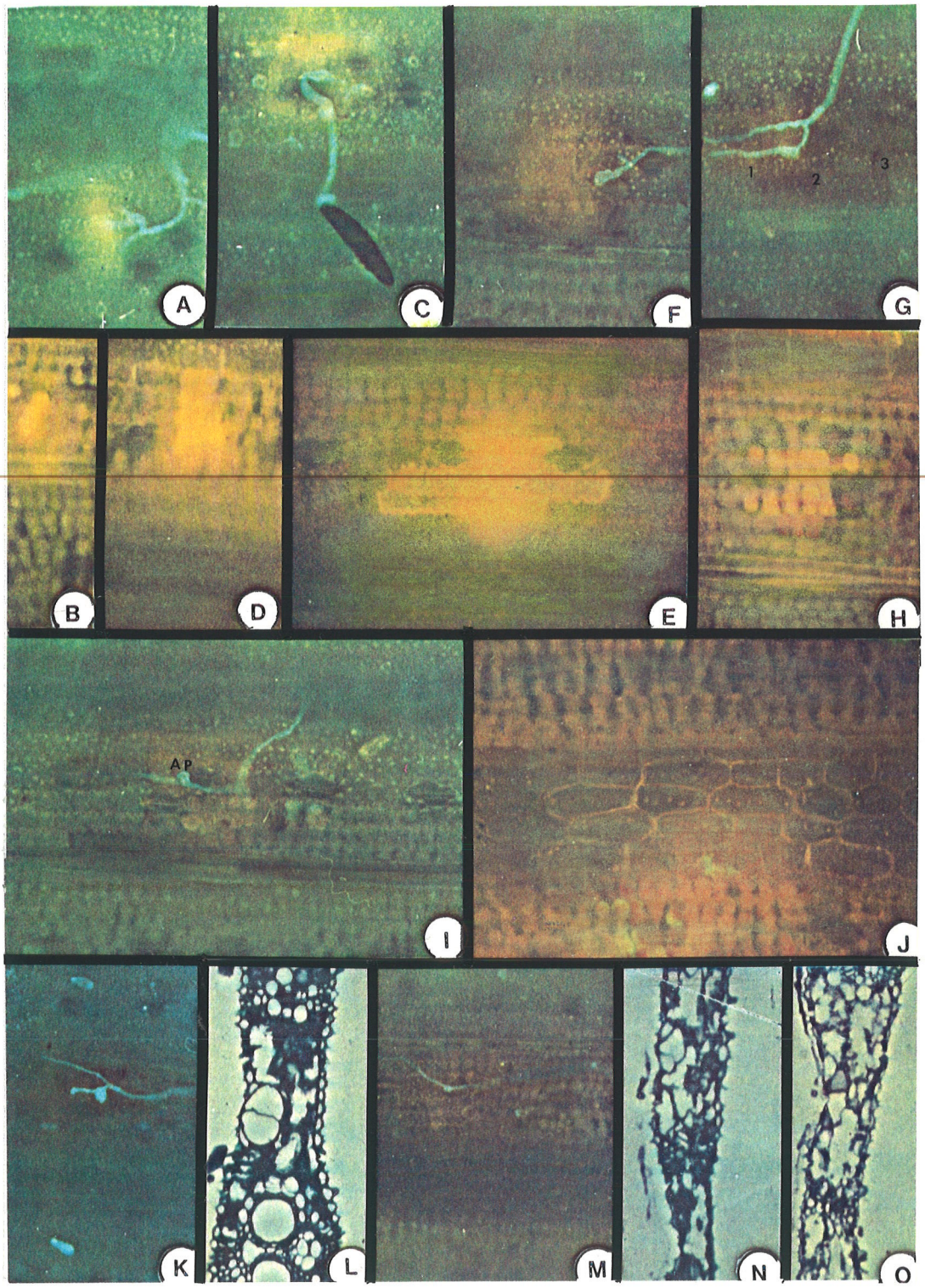
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Fig. 13. Histology of wild rice aerial leaves infected by *Helminthosporium oryzae*.

72 hours post-inoculation (A-C)

A) Intercellular invasion of mesophyll cells with resultant wall swelling and cytoplasmic degeneration. Intracellular invasion of bundle sheath and sclerenchyma cells has also occurred (MB;-). x 529.

B) Intracellular hyphae in xylem parenchyma cells (MB;-). x 1380.

C) Autofluorescence of host cells in a lesion with no visible Calcofluor-stained hyphae (C;Fl-I). x 254.

7 days post-inoculation (D-I)

D) Destruction of mesophyll cells and alteration of bulliform cell walls in a discrete lesion (MB;-). x 529.

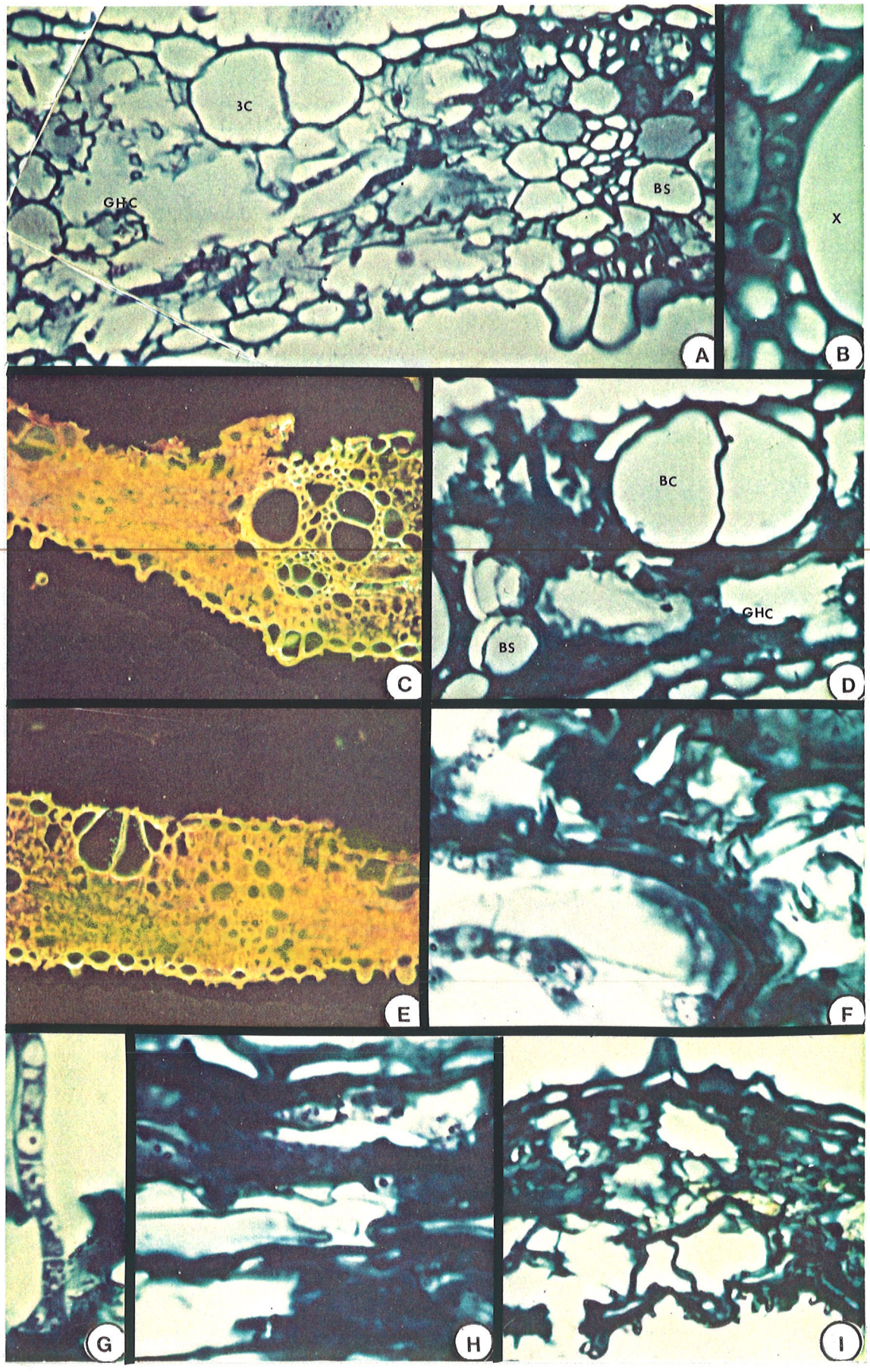
E) Haloed lesion with intense autofluorescence of host cells at the centre diminishing towards the periphery (C;Fl-I). x 254.

F) Destruction of mesophyll cells by hyphae in necrotic coalesced lesion showing total disruption of cellular contents. Note change of color of host tissues (MB;-). x 1380.

G) Hyphal element with well-defined septae and cytoplasmic components in severely damaged mesophyll tissue (MB;-). x 1380.

H) Epidermal cells are not invaded, but develop thick swollen cell walls possibly due to the influence of hyphae in the underlying mesophyll tissue (MB;-). x 1380.

I) Total destruction of the mesophyll and epidermal cells by the pathogen with a coalesced lesion. Note the accumulation of yellow-brown material which gives an overall greenish tint to the host tissue (MB;-). x 529.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induced a yellow autofluorescence of guard and underlying mesophyll cells (Fig. 11, E,F). Infection hyphae entered the sub-stomatal cavity causing damage to the guard cells, subsidiary cells, and over-arching papillae, as well as the underlying mesophyll cells (Fig. 11, G).

Penetrations in the stomatal band were quite common; the infection hyphae apparently penetrating through interstomatal cell wall junctions. The hyphae often invaded the lumina of these epidermal cells causing their walls to swell and turn brown. Papillae of all affected cells turned brown only at the base and remained unchanged at the tip (Fig. 11, G,H). Costal cells were rarely penetrated; however infection hyphae invaded sclerenchyma via interstomatal cells. Infection hyphae progressed intracellularly and intercellularly in areas of sclerenchyma and then invaded the bundle sheath (Fig. 11, I). The bands of bulliform cells were less frequently penetrated by the pathogen. Initially appressoria formed over the bulliform cells and often adjacent cell walls showed a slight yellow autofluorescence (Fig. 11, J-L). Multiple invasion of bulliform cell walls by branches of a germ tube were even rarer, although the bright yellow autofluorescence of cell walls and junctions was the same (Fig. 11, K-M). In sectional view, invasion of cell junctions appeared to be the principal mode of fungal entry, followed by colonization of adjacent cells. A few cells responded to penetration with granulation of the cytoplasm (Fig. 11, N), whereas most only showed browning and swelling of cell walls. After entry into the epidermis, hyphae proceeded intercellularly to infect mesophyll cells (Fig. 11, O).

Helminthosporium species generally penetrated host epidermal

cells directly through cell wall junctures or through stomates (Healy and Britton, 1968; Jennings and Ullstrup, 1957; Knox-Davies, 1974; Chui et al., 1972; Hau and Rush, 1979; 1982). Penetration of rice by H. oryzae was predominantly (95%) by way of bulliform cells and only rarely (5%) stomatal (Ou, 1972; Hau and Rush, 1982), whereas in wild rice no single route was favored. Hau and Rush (1982) reported that appressoria formed mainly over bulliform cells, and citing Whitney (1976) suggested that outer bulliform cell walls remain in a relatively soft pectic-cellulosic state long after other epidermal cells have become lignified. The outer walls of wild rice bulliform cells (Fig. 5, H) stained differently from those of other lignified epidermal cell walls. However, even if they were softer there was no indication that they afforded the same favorable site of penetration as those of rice. Horsfall and Cowling (1979) have proposed that appressorial formation was primarily controlled by pathogen genotype and it may be that isolate FA 72 D#5 on wild rice stock plants had no preference for bulliform cells. Yadav (1981) has suggested that H. sativum cannot penetrate the host without exogenous nutrients in the form of leaf exudates. Rice leaf exudates may have been more abundant or stimulatory to H. oryzae than were those of wild rice.

c. Lesion formation and development

Small lesions emitted a bright yellow autofluorescence and were usually circular in appearance (Fig. 12, A-J). In the early stages of infection, it was possible to count the number of infected mesophyll cells and in turn follow the progress of infection. The walls of epidermal cells overlying lesions autofluoresced yellow (Fig. 12, E). The next stage was the formation of a band or ring of reddish brown

pigmented cells around the autofluorescent host cells. Brown pigmentation increased in cells adjacent to the band, however, cells which were near the initial penetration site remained yellow. Gradually, the brown pigmentation spread to the vein (Fig. 12, I). Bulliform cells occasionally developed red pigmentation. Eventually, at about 72 h p.i., lesion types became visible to the unaided eye. Discrete lesions (Fig. 12, K,L) appeared limited by vascular bundles and were densely pigmented without any central yellow host cells. Large lesions, either coalesced or haloed, showed extensive browning in all related cells except those near the penetration point (Fig. 12, M-O). Coalesced lesions in transverse section were shrunken and collapsed (Fig. 12,N). Haoled lesions were similar to coalesced lesions with less shrinkage, but with extensive damage in central necrotic regions (Fig. 12, O). These lesion types may be compared to those shown in Fig. 1.

The primary gold to yellow fluorescence emitted by infected wil rice aerial leaf cells is a phenomenon of necrotic host cells responding hypersensitively to rust and powdery mildew fung (Marte and Montalbini, 1972; Mayama and Shishiyama, 1976a; 1978; Rohringer et al. 1977; Kita et al., 1980). Marte and Montalbini (1972) concluded that a characteristic yellow fluorescence was one of the earliest detectable signs of alteration of host cells and that it coincided with phenol accumulation. This was confirmed by Mayama and Shishiyama (1978) and Kita et al. (1980) who reported that the autofluorescent and U.V.-absorbing substances which accumulated were polyphenolic compounds. Such compounds may also be the cause of the yellow autofluorescence in infected wild rice aerial leaf cells.

The histology of the infected host cells is given in Fig. 13.

After penetration of epidermal cells, the fungal hyphae proceeded intercellularly through the mesophyll area. Arm-type parenchyma cells, at the periphery of the lesion, underwent wall thickening, degeneration of organelles and granulation of the cytoplasm. In some cases, cellular integrity was totally lost. Colonization then became extensive; bundle sheath cells were invaded by intracellular hyphae and often contained granular cytoplasm (Fig. 13, A). Fig. 13, B shows hyphae in the vascular bundle, including the xylem and phloem. The spread of the pathogen from a necrotic area bounded by vascular bundles into a new region of intercostal mesophyll appeared to be by way of bundle sheath or sclerenchyma cells rather than directly through vascular tissue. Epidermal cells in these areas were not normally invaded (Fig. 13, C).

Host cells in discrete lesions soon became necrotic. Although hyphal growth appeared limited by bundle sheath cells, some cells on the other side of the vascular bundle autofluoresced and showed signs of disruption (Fig. 12, L and Fig. 13, D) providing evidence of pathogenesis in advance of hyphal colonization.

The central necrotic areas of large coalesced lesions were indicated by bright yellow autofluorescence (Fig. 13, E). Within these areas, mesophyll cells were totally disrupted and collapsed. Vascular bundles were shrunken and the entire wall of their cells autofluoresced strongly. This is confirmed in Fig. 13, F, which shows the complete disorganization and bluish-green staining of the arm-type parenchyma. Contrary to the effect of fungal invasion in the mesophyll and vascular bundles, epidermal cells appeared to be relatively undisrupted, even when in contact with hyphae (Fig. 13, H).

Even in areas of necrosis, invading hyphae appeared unaffected by host contact and showed septa and discernible organelles (Fig. 13 A,G). In contrast, hyphae in the discrete lesion appeared thinner and lacked obvious organelles (Fig. 13, D). Conidial vigor, in some cases may be diminished, which may have allowed the host cells to defend themselves more ably or more quickly, thus confining the spread of the pathogen. Coalesced and haloed lesions may result from the combined penetration and spread of several vigorous conidia. By 7 days p.i., tissue destruction can be virtually complete in large coalesced lesions (Fig. 13, I).

The observable symptoms may be induced, at least in part, by the phytotoxin, ophiobolin which was detected at the site of H. oryzae invasion of rice leaves (Narain and Simmachalam, 1975) and which produced membrane disruption and changes in respiration, protein synthesis and nucleic acid synthesis (Chattopadhyay and Samaddar, 1980a). However, the general disruptive effect on host cells is related not only to ophiobolin, but also to the accumulation of yellow fluorescent phenolics and to the enzyme, polyphenol oxidase, PPO, as discussed in the current hypothesis for the development of brown spot symptoms proposed by Chattopadhyay and Samaddar (1980b). Assuming that isolate FA 72 D#5 of H. oryzae used in this experiment releases a toxin, then their hypothesis would account for the appearance of yellow autofluorescence of cells and also for the disruption of host cells (Figs. 12 and 13). As indicated in Fig. 11, penetration of the host by H. oryzae can be through stomates or through cell wall junctions. Following penetration by these routes, hyphae may produce ophiobolin and PPO which cause membrane disruption and many other distortions

in mesophyll cells adjacent to those initially affected. This would account for the continuing yellow autofluorescence of cells at the initial penetration point and the browning of cells surrounding it (Fig. 12). The browning and swelling of epidermal cell walls appears to be secondary in that PPO and phenolics released from moribund meosphyll cells underlying the epidermal cells could affect the cell walls while leaving the internal cell morphology relatively unaffected. The fact that non-invaded epidermal and mesophyll cells show browning and swollen walls indicates that release of ophiobolin and/or PPO precedes colonization of new cells. Lesions would develop dark brown pigmentation after the phenolics reached a concentration high enough for their oxidation products to begin to accumulate. It may be that lesion size is determined by production of a given amount of ophiobolin per successful penetration by H. oryzae. Lesion size would then be related to conidial vigor and also to the susceptibility of the host cells to ophiobolin. Discrete lesions would result when conidial vigor is diminished possibly in conjunction with a faster response to invasion by the host cells. Coalesced lesions would result from several successful penetration and invasion events.

The infection of wild rice aerial leaves by H. oryzae had many features in common with other host-pathogen interactions. Tullis (1935) reported the same intercellular and intracellular colonization of rice by H. oryzae as was seen in wild rice. The symptoms described by Horino (1973) at the light microscope level were similar to those presented in this study. At the ultrastructure level, he suggested that nuclei, chloroplasts and mitochondria, as well as lipid bodies and starch were all affected by contact with the pathogen. Changes

in lipid bodies were also noted by Sridhar et al. (1973). The similarities in leaf morphology and gross symptoms would lead one to expect similar changes at the ultrastructural level in wild rice, especially in view of the known effect of ophiobolin on menbrane permeability. Many Helminthosporium species have been shown to produce phytotoxins (Heitefuss and Williams, 1976), for example H. maydis and H. carbonum (Jennings and Ullstrup, 1957; Murray and Maxwell, 1975) and also H. sorokinianum (Healy and Britton, 1968), which may account for observations of similar symptoms at the mesophyll cell level.

One difference between the effect of facultative saprophytes such as H. oryzae and obligate pathogens such as Puccinia graminis Pers. appears to be in the extent of the autofluorescence of affected host cells. H. oryzae causes an immediate 22-24 h p.i. yellow autofluorescence of host cells near the initial penetration site, which gradually becomes a brown area as final lesion size is achieved. P. graminis causes yellow autofluorescence of resistant host cells (Rohringer et al., 1977) in a manner similar to that of H. oryzae on wild rice susceptible cells. However, the affected wheat cells in resistant varieties succumb immediately and turn brown individually; no large coalesced lesions are formed (Rohringer et al., 1977; Samborski et al., 1977). It could be that yellow autofluorescence, representing the build up of oxidized phenolic products is a universal phenomenon displayed in host-pathogen interactions involving facultative saprophytes. The response in hosts resistant to obligate pathogens may simply be an evolutionary remnant of this phenomenon. Although reports on the development of other Helminthosporium species in their respective hosts have correlated invasion with host necrosis, this study

and that of Stockwell and Sherwood (1981) represent the first association between yellow autofluorescence and facultative saprophytes, and further studies may confirm the universality of this phenomenon.

d. Sporulation

The final stage in the disease cycle was sporulation of H. oryzae on aerial leaf lesions. Sporulation of the pathogen did not occur until leaves had become senescent about six weeks p.i. Initially small greyish areas developed over the lesions and upon examination, it was found that they consisted of immature conidia. These matured into brown-pigmented conidia. The stages of conidial formation were as described by Fukatsu and Kakizaki (1955), for H. oryzae on rice. Sporulation of the pathogen on the aerial leaves corresponded with that on the floating leaves which is described later.

e. Evaluation of histological techniques

Clearing whole leaf mounts with lactophenol/alcohol had no detrimental effect on germ tubes or host tissue. Of the 12 fluorochromes tested for use with germ tubes, Calcofluor, ethidium bromide (EB), Auramine O, acriflavine, picridine orange, Coriphosphene O and aniline blue (AB) were taken up readily by the germ tube walls. Nile Blue A primuline, congo red and Rhodamine B did not cause H. oryzae germ tubes to fluoresce with the available filter systems. Only Calcofluor and EB provided sufficient contrast for satisfactory photomicrography (Appendix II).

The best differentiation between germ tubes and host cells was achieved by the use of Calcofluor. Germ tubes fluoresced brilliant blue using either filter system 1-I or 3-I (Fig. 11, C,D). Appressoria were also brilliant blue and the various shapes could be easily discerned.

Calcofluor fluorescence lasted about 30 days if tissue was prepared by method #1 (see methods and materials), and this method proved excellent for generalized examination of leaf segments. However, method #2 ensured sharper contrast for photomicrography. Calcofluor has been used extensively to fluoresce infection structures of various rust fungi (Patton and Johnson, 1970; Rohringer et al., 1977; Samborski et al., 1977; Kuck et al., 1981), and also H. sativum (Stockwell and Sherwood, 1981). The success of Calcofluor as an agent for discerning infection structures has been attributed to its affinity for cellulose, chitin, and other β-linked polymers (Maeda and Ishida, 1967; Harrington and Raper, 1968; Hayashibe and Katohda, 1973), all of which have been found in H. oryzae germ tube walls (Nanba and Kuroda, 1971 a,b,c).

EB concentration #1 (0.001%) stained the germ tube contents, branches and appressoria a uniform yellow-orange or orange-red color (Fig. 11, A), while the higher concentration #2 (0.005%) stained conidial nuclei and germ tubes bright red (Fig. 11, B). The usefulness of the effect was limited by the quick-fading nature of the stain. Although, many fungal species have been stained effectively with EB (Calich et al., 1978; Roser, 1980; Roser et al., 1982), only one study deals with host-pathogen differentiation (Kuck et al., 1981). Although acridine orange was shown to be translocated between conidia of powdery mildew and infected barley cells via the pathogen germ tubes (Kunoh and Ishizaki, 1981), it could not be used effectively in this study.

Methylene blue (MB) allowed the differential visualization of H. oryzae hyphae, infected host cells and healthy host cells in thin sections of infected aerial leaf tissue which had been embedded in JB-4

plastic. Infected host tissue appeared bluish-green in contrast to its healthy counterparts; hyphae appeared purple (compare Figs. 5 and 11). A 3-dimensional reconstruction of the penetration process was made possible by combining observations of surface events made using epifluorescence with those of internal events from conventional light microscopy. The bright yellow autofluorescence of the phenolic compounds in affected host cells correlated well with their bluish-green color with MB. MB has been used to stain hyphae of H. oryzae infecting O. sativa (Tullis, 1935). Toluidine blue and trypan blue did not give adequate differentiation of H. oryzae hyphae in wild rice aerial leaves, and neither did acid fuchsin/toluidine blue, used previously to stain healthy aerial leaf thin sections.

C. HOST-PATHOGEN INTERACTION #2: Wild rice floating leaves/*H. oryzae*

1, Histology of *Zizania aquatica* - the healthy host

a. Gross anatomy

Major differences between wild rice aerial and floating leaves involved the epidermal cells and their walls (compare Figs. 5 and 14). Floating leaf epidermal cells bore numerous small papillae which were especially prominent on the larger papillae of the adaxial surface (Fig. 14, A,I,J). In contrast to the aerial leaves, the upper and lower epidermis of the floating leaves proved to be strongly dissimilar. Comparison of Fig. 5, B and Fig. 14, B showed that the adaxial surface of the floating leaf resembled that of the aerial leaf. However, the abaxial surface was composed of long cells devoid of papillae, stomates, and epidermal hairs (Fig. 14, D). One common feature of both surfaces was the presence of *Oryza*-type silica bodies, similar to those found on aerial leaves. Although the adaxial surface resembled that of aerial leaves, large papillae were more prominent and bulliform cell zones were less so. Stomates appeared identical on the two leaf types, however trichomes were more apparent on cells in the stomatal band of the floating leaves (Fig. 14, B). The strong affinity for Calcofluor of large papillae and bulliform cells (Fig. 14, E,F) suggested that their walls may differ chemically from other parts of the epidermis.

The floating leaf mid-rib was much less prominent than that of the aerial leaves, and the epidermal cells overlying it appeared quite different from their aerial leaf counterparts (compare Figs. 14,G and 5,F). The floating leaf mid-rib had large lacunae on either side of a large vascular bundle and a smaller vascular bundle above the large one (Fig. 14, G).

Fig. 14. The anatomy of wild rice floating leaves.

Adaxial surface (A-C)

A) Large and small papillae on the outer walls of epidermal cells (AB;F2-I). x 800.

B) Bulliform cells, stomates, silica bodies and interstomatal cells (AB;F2-I). x 800.

C) Nuclei of cells (EB;F3-II). x 600.

Abaxial surface (D)

D) Long cells and silca bodies; stomates and bulliform cells lacking (AB;F2-I). x 800.

E) Calcofluor-fluorescence of large and small papillae on the adaxial surface (C;F1-I).

Transverse sections (F-J)

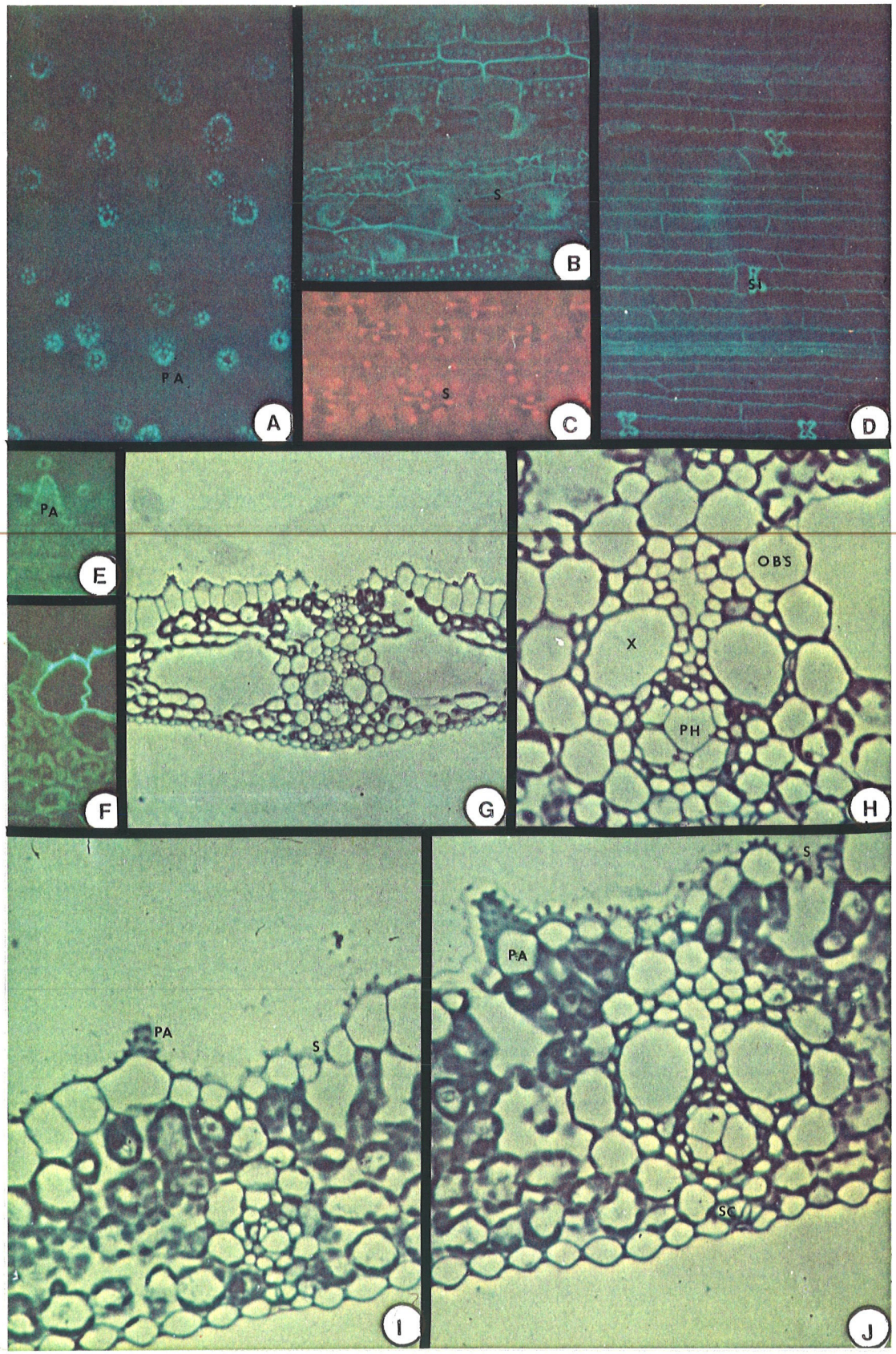
F) Calcofluor-fluorescence of bulliform cell walls (C;F1-I). x 508.

G) Mid-rib showing large central vascular bundle flanked by two lacunae; small adaxial vascular bundle; strong distinction between upper and lower epidermis (AF/TB;-). x 154.

H) Large vascular bundle with outer bundle sheath cells containing chloroplasts, thick-walled inner bundle sheath cells, xylem and phloem (AF/TB;-). x 508.

I) Lamina showing papillate cell walls of adaxial epidermis, smooth abaxial epidermial cell walls, stomatal complex, small vascular bundle, lack of fusoid cells, arrangement of mesophyll cells (arm-type parenchyma) and their parietal chloroplasts (TB;-). x 508.

J) Lamina showing epidermal cell types and walls overlying a larger vascular bundle with double bundle sheath and associated sclerenchyma (TB;-). x 508.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The vascular bundles of aerial and floating leaves were similar (Fig. 14, H), except that large fusoid cells did not occur in association with any of the vascular bundles of the floating leaves (Fig. 14, I,J). The only lacunae in the floating leaves were those associated with the main vascular bundle of the mid-rib.

The differences in the epidermal cells observed in the surface view were reflected in the transverse sections. The adaxial epidermis consisted largely of more palisade-like and deeper cells. Smaller cells were associated only with stomata or larger veins. Cells of the abaxial epidermis were thin-walled and extremely uniform. Chloroplasts of bundle sheath and mesophyll cells appeared to be similarly located in aerial and floating leaves. These are seen in Fig. 14, I,J.

A comparison of Figs. 5 and 14 indicates the similarities in internal structure of the two leaf types.

The only pertinent report on the wild rice floating leaves (Z. aquatica var. angustifolia) is by Weir and Dale (1960). They referred to the small papillae on the adaxial epidermal cell walls as blisters and suggested that these produced a roughness which trapped air bubbles and aided in creating a non-wettable surface.

b. Evaluation of histological techniques

Techniques for examining healthy floating leaves produced some different results when applied to aerial leaves. Comparing Fig. 5, B,D with Fig. 14, A,B,D, it was found that AB-treated floating leaf cells were better differentiated when filter system F2-I was used rather than F2-II, as preferred for aerial leaf use. EB produced no staining differences (Fig. 14, C). Because Calcofluor showed less affinity for

cell walls of floating leaves than aerial leaves, for superficial anatomical studies, it proved less useful than AB, although in the case of JB-4 plastic-embedded material, the affinity of Calcofluor was similar on the two leaf types (Fig. 14, F).

Toluidine blue alone or acid fuchsin/toluidine blue (Feder and O'Brien, 1968) were most effective in differentiating the various cell types in JB-4 plastic-embedded material (Fig. 14, G-J). Adaxial epidermal cells appeared to stain a darker blue than their aerial leaf counterparts and abaxial cell walls a lighter blue. Cells of the vascular bundle and mesophyll area stained in the same manner as those of the aerial leaves, indicating again the similarity of the internal histology. However, the ease with which aerial leaves were sectioned using JB-4 plastic was not duplicated when sectioning the floating leaves. The abundant surface silica bodies on both surfaces of the floating leaves quickly damaged the edge of a glass knife which made serial sectioning very difficult.

At the ultrastructural level (Figs. 15 and 16), all cells appeared to have a slight "peppering" or precipitate in them. However, most of the characteristics identified by Hayat (1970) as indicators of adequate preparation were observed. Cell walls of epidermal cells showed granulation, except the thinner walls associated with guard and subsidiary cells (Fig. 15, A,B,C). Sclerenchyma cells which lie above and below vascular bundles (Fig. 14, J) appeared similar using either procedure 3 or 5. Chloroplasts of floating leaf bundle sheath cells (Fig. 15, D) and mesophyll cells responded similarly to those of aerial leaf cells (Fig. 9, A). The floating leaf ultrastructure appeared normal when procedure 5 was used (compare Figs. 16 , A and 6, C). The

Fig. 15. Ultrastructure of epidermal, sclerenchyma and bundle sheath cells of wild rice floating leaves.

A) Thick granular layered papillae extending from generally thin wall of an epidermal cell. x 6300.

B) Stomatal complex. x 8000.

C) Junction of two epidermal cell walls showing granular layering. x 12 000.

D) Sclerenchyma cells above bundle sheath (procedure 3). Chloroplasts in bundle sheath cells contain larger osmiophilic globules and less defined thylakoid structure than by procedure 5. x 12 000.

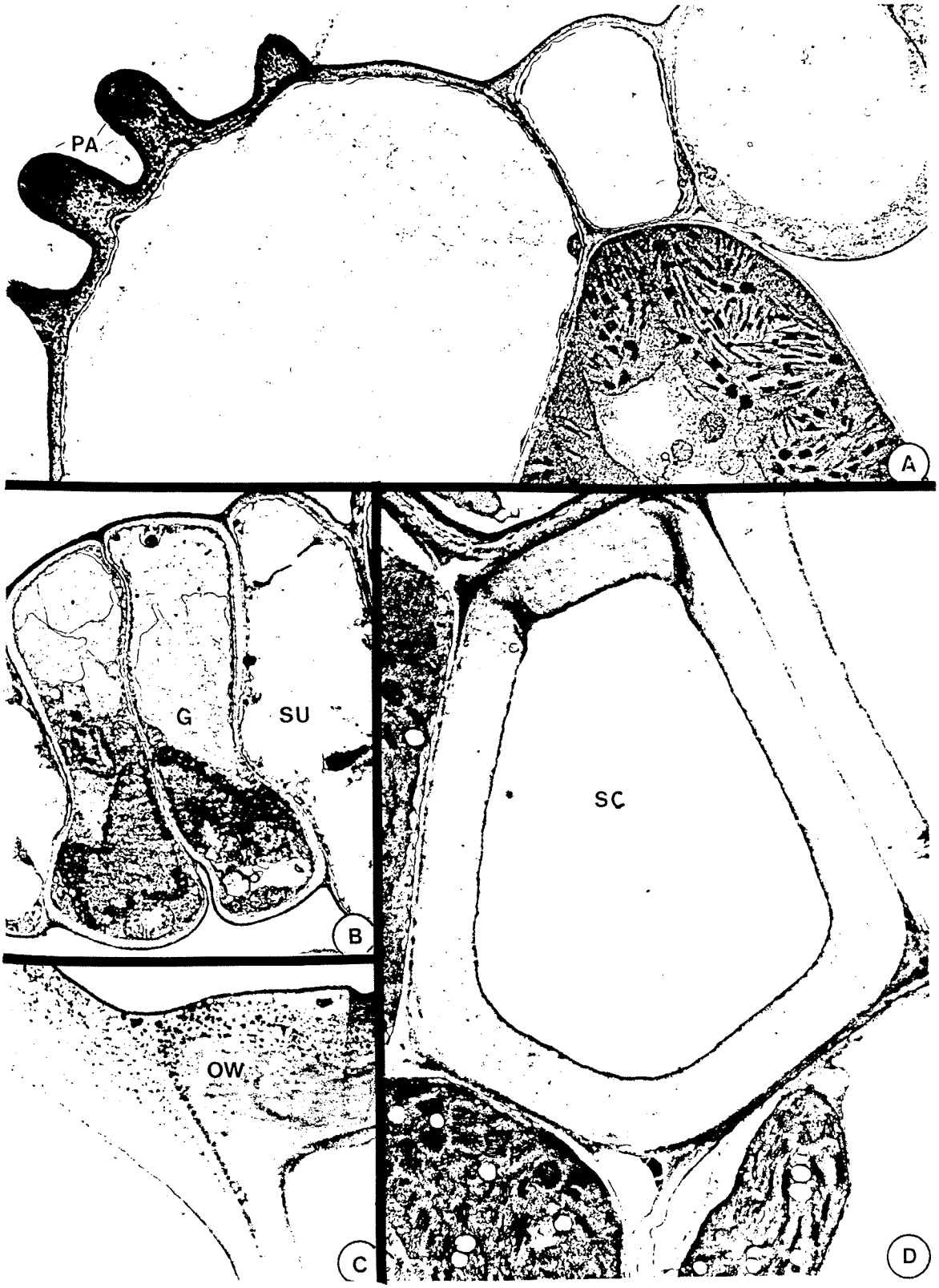
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Fig. 16. Ultrastructure of mesophyll (arm-type parenchyma) cells of wild rice floating leaves.

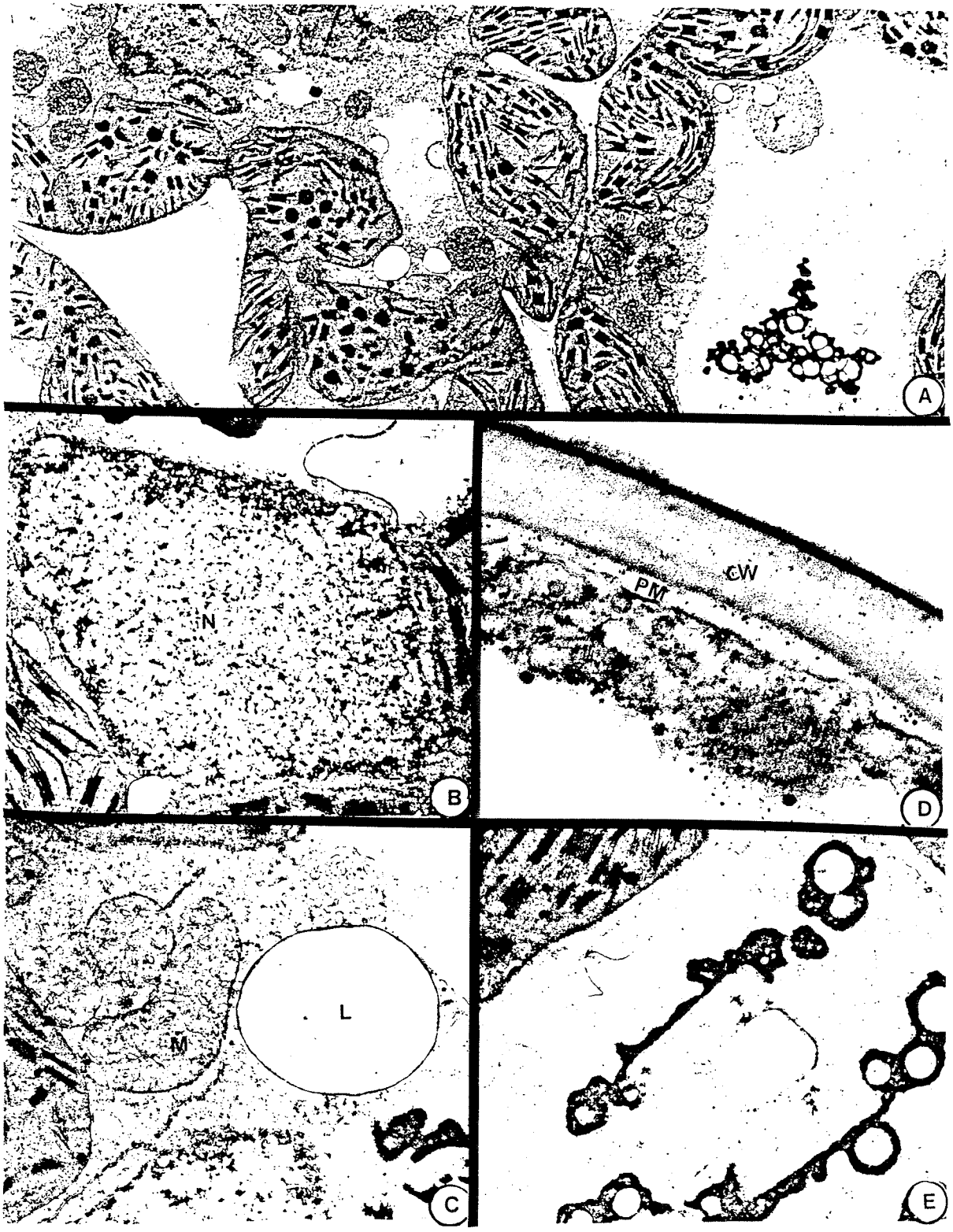
A) Overview showing invaginated cell wall and the relative positions of the organelles. Note some peppering throughout. x 6300.

B) Nucleus. x 15 000.

C) Mitochondria and lipid body in association with a chloroplast. x 18 000.

D) Cell wall and plasma membrane showing adverse effect of acrolein in fixative (procedure 2). x 50 000.

E) Characteristic aggregations of electron dense circular or spherical bodies. x 15 000.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double membrane of the nuclear envelope was intact and the nuclear contents were uniformly dense with masses of chromatin (Fig. 16, B).

Floating leaf cell walls and membranes suffered some damage when prepared by procedures 1 and 2 (Fig. 16, D). This may have been an effect of the acrolein, which could cause a very pronounced decrease in extractable lipid (Hayat, 1970), or it may simply have been the result of poor initial fixation. Mitochondria appeared neither swollen nor shrunken, if procedure 5 was used, however, the clarity of the membranes in both mitochondria and chloroplasts was obscured by the peppering effect (Fig. 16, C), which was probably due to inadequate washing of the leaves by the aqueous phosphate buffer. A procedural criticism was that large areas within most mesophyll cells had what may be artifacts of poor fixation or precipitated components of these cells (Fig. 16, A,C,E). The reason for problems arising from procedure 5 as applied to the floating leaves is explained in the General Discussion. The details of the electron microscopy procedures are given in Appendix III and techniques which are recommended for use with floating leaves are summarized in Appendix IV.

c. Ultrastructure of healthy cells

The differences between epidermal cells and the similarities in bundle sheath and mesophyll cells in the two leaf types extended to the ultrastructural level. Epidermal cells were again highly vacuolate but the walls were much thinner in floating leaves (Fig. 15, A). Fig. 15, B shows the similar chlorophyll-bearing guard cells (compare Fig. 7, A). That papillae were merely extensions of the cell wall was confirmed by the continuity of the granular layers (Fig. 15, C).

Sclerenchyma cells (Fig. 15, D) were identical in both aerial and floating leaves. The chloroplasts characteristic of bundle sheath cells of aerial leaves were also present in the floating leaves (Fig. 15, D). As discussed earlier, this may indicate that wild rice could be a C-4 plant, but physiological studies are needed to confirm this.

Mesophyll cell organelles were generally similar in type and orientation to those of aerial leaves (Fig. 16, A,C). On the other hand, large internal areas often lacked typical organelles, but characteristically contained aggregations of electron dense circular or spherical bodies (Fig. 16, A,E). Nuclei of mesophyll cells (Fig. 16, A,B) did not appear associated with lipid bodies as did those of the aerial leaves (Fig. 8, C); indeed the lipid content appeared considerably less than that of the lipid-rich aerial leaf cells. Whether the aggregations shown in Fig. 16, E were natural or artifacts of preparation remains unclear at present. The vascular bundles of the two leaf types were similar.

Ultrastructurally and morphologically, floating leaves are similar to aerial leaves, except for the epidermis. Therefore, pathogens such as Helminthosporium oryzae could be expected to encounter the same kinds of internal organization in the floating leaves as in the aerial leaves after penetration has occurred.

2. The disease cycle

The stages of the infection process on the floating leaves of wild rice infected by Helminthosporium oryzae are presented in Figs. 17 and 18.

a. Pre-penetration

Germination of conidia and appressorium formation showed the same patterns on floating leaves as those described previously for aerial leaves. The higher concentration of conidia on floating leaves resulted in inhibition of appressorium formation and penetration (Fig. 17, A), perhaps due to competition for exogenous nutrients (Yadav, 1981). Calcofluor and EB were again effective in differentiating superficial fungal infection structures. The background presented by the floating leaf adaxial epidermis was particularly advantageous in the case of EB (Fig. 17, B).

b. Penetration

As with aerial leaves, penetration of floating leaves by pathogen appressoria was associated with bulliform cell and stomatal bands. Infected cells of the stomatal complex fluoresced greenish-yellow with EB and filter system 3-II (Fig. 17, B) and bright yellow using the Calcofluor technique (Fig. 17, C,D). It may be that the UV illumination excited the fluorescent cellular contents of the underlying mesophyll cells through the thinner epidermal cell walls to give the better differentiation of the autofluorescent host cells in the floating leaves, when compared to those of aerial leaves (compare Figs. 12 and 17).

c. Lesion formation and development

Lesion formation in floating leaves proceeded through similar

Fig. 17. Penetration and initial lesion development on wild rice floating leaves infected by Helminthosporium oryzae.

Approximately 24 hours post-inoculation (A-D)

A) Cluster of conidia showing germ tube development but suppression of appressorium formation and penetration (C;F3-I). x 600.

B) Stomatal penetration and infection by germ tube with appressorium (EB;F3-II). x 600.

C) Stomatal penetration (C;F1-I). x 800.

D) As C), but showing only autofluorescence of affected epidermal and mesophyll cells (C;F1-II). x 800.

48 hours post-inoculation (E-M)

Small lesion in stomatal band (E-G)

E) Germ tube surrounding large epidermal papilla and forming appressorium (C;F1-I). x 360.

F) Autofluorescent bulliform cell (C;F1-II). x 360.

G) Autofluorescent mesophyll cells (C;F1-II). x 360.

H) Small lesion at the level of the epidermis showing autofluorescence of affected cell walls and of underlying mesophyll cells. Note distorted appearance of the stomate. (C;F1-I). x 800.

I) As H), but showing only the autofluorescence of epidermal cell walls and of underlying mesophyll cells (C;F1-II). x 800.

J) Longitudinal expansion of a small lesion showing a change in color of fluorescence to yellow-orange (C;F1-I). x 466.

K) Normal pattern of bulliform cell infection. Walls of affected cells autofluoresce greenish-yellow (EB;F3-II). x 466.

L) Autofluorescent cells adjacent to and underlying a bulliform cell penetration site (EB;F3-II). x 466.

M) Wall autofluoresce of affected long cells (compare to K) (EB2;F3-I). x 466.

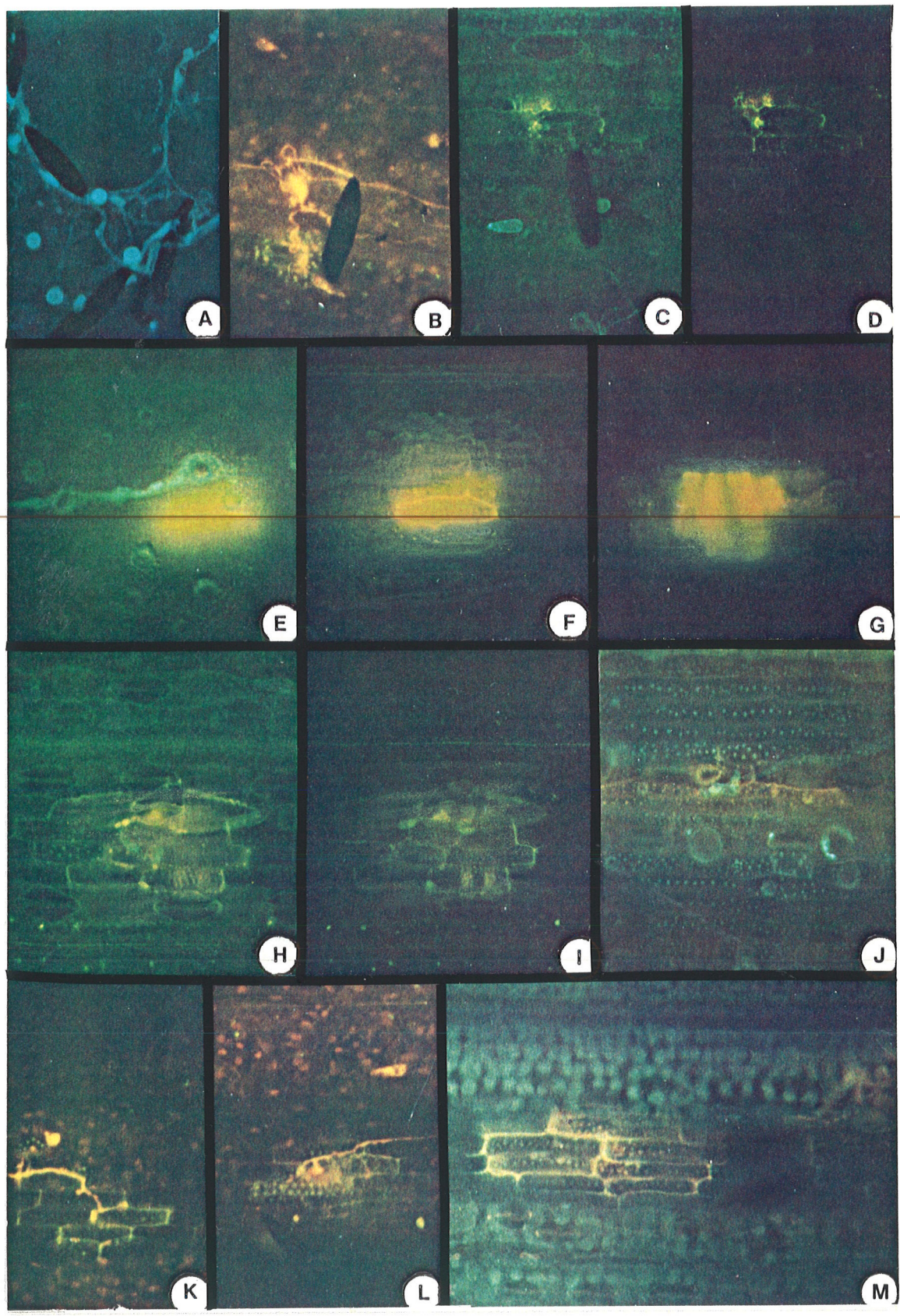
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Fig. 18. Lesion development on adaxial and abaxial surfaces of wild rice floating leaves infected by Helminthosporium oryzae.

48 hours post-inoculation - adaxial surface (A-B)

A) Longitudinal expansion of lesion with slight darkening of autofluorescence but no visible indication of a brown delimiting band (C;Fl-I). x 260.

B) As A), but showing extensive golden-yellow autofluorescence of affected host cells (C;Fl-II). x 260.

Brown-ring lesion type (C-F)

C) Overview showing characteristic pigmented peripheral ring surrounding tissue containing blue-fluorescent hyphae (C;Fl-I). x 135.

D) Area near dark peripheral ring showing the nature of the ring cells, yellow autofluorescent host cells and cells containing blue-fluorescent hyphae (C;Fl-I). x 300.

E) Central area showing hyphal elements and autofluorescent host cells (C;Fl-I). x 468.

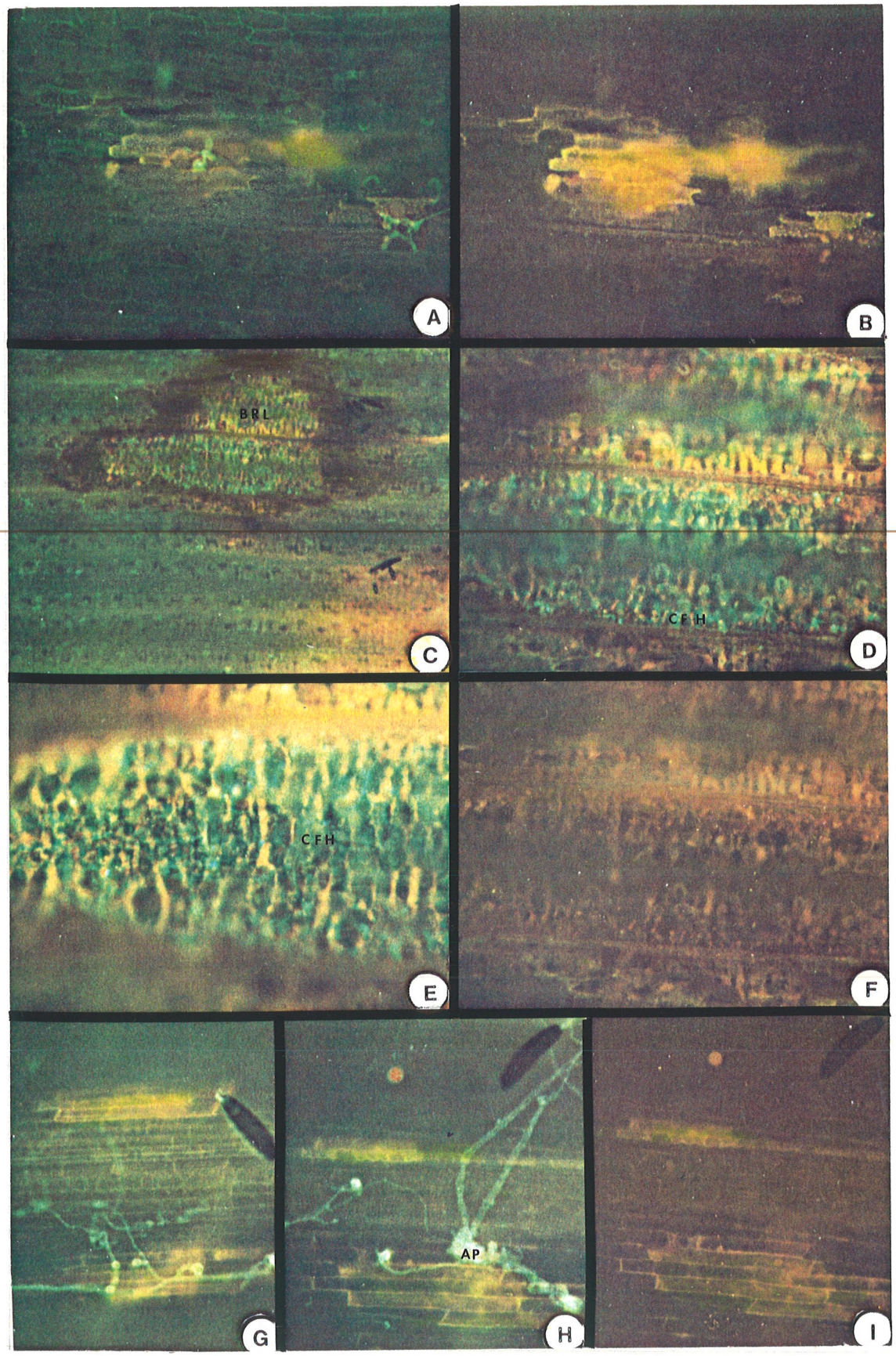
F) Central area showing hyphal elements and autofluorescent host cells (C;Fl-I). x 300.

Abaxial surface (G-I)

G) Longitudinal expansion of small lesions (C;Fl-I). x 340.

H) Autofluorescence of cells adjacent to sites of initial penetration in a larger lesion (C;Fl-I). x 480.

I) As H), but showing only autofluorescence of eipdermal cell walls and of underlying mesophyll cells (C;Fl-II). x 480.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stages to those of aerial leaves; however a comparison (Figs. 12, 17 and 18) showed that the principal differences were: (1) floating leaf lesions tended to expand in a linear pattern in contrast to the more circular pattern found on aerial leaves; (2) color changes from yellow to brown (in discrete and coalesced lesions) took place more gradually and the reddish-brown cells found in the aerial leaves were less prominent; and (3) characteristic brown-ringed lesions developed (Fig. 18, C-F). The brown-ring lesion types, not seen on the aerial leaves, may simply represent some differential gene expression at the floating leaf stage. It is not uncommon for different leaf types to express differences in resistance, for example, juvenile resistance in cereals to certain pathogens (Jones and Clifford, 1978).

The autofluorescence of infected host cells (Fig. 17, E-M, Fig. 18 A,B) was not only more intense than in aerial leaves, but also remained in evidence at 48 h p.i., at which time it was quenched in aerial leaf lesions due to the accumulation of brown pigments (Fig. 12, K,M). While aerial leaves developed visible lesions at 18-24 h p.i. and fully developed ones at 33-36 h p.i., lesions on floating leaves were still generally small to medium-sized at 48 h p.i. Thus lesion development on floating leaves appeared to be delayed approximately 16-20 h, as compared with aerial leaves. These differences in pathogen behavior towards the two leaf types are explained fully in the General Discussion.

Although some differences in pathogen behavior were noted, the similarities in the two interactions with regard to pre-penetration, penetration and host cell infection emphasized the importance of the floating leaves as potential sites of colonization by H. oryzae, prior to the severe and often sudden late summer outbreaks of Helminthosporium

blight. That floating leaves were indeed infected by H. oryzae was evident in that infected host cells showed a bright yellow autofluorescence (Figs. 17 and 18), a phenomenon which indicated infection of aerial leaf cells. Also, lesion types were similar on both leaf types, with the exception of the brown-ringed lesions. The pattern of infection by way of bulliform and long cell walls indicated by cell wall autofluorescence corresponded with that of the aerial leaves (compare Figs. 11, L,M and 17, K-M). Internal autofluorescence of bulliform cells only occurred after they had been invaded (Fig. 17, H); the cytoplasm of corresponding cells in aerial leaf transverse sections appeared granular (Fig. 11, N). Finally, the pattern of penetration and colonization of long cells of the floating leaf abaxial epidermis (Fig. 18, G-I) appeared to be similar to that of bulliform and long cells of the adaxial surface, and also to that of the aerial leaves.

Lesions did develop on the abaxial surfaces of floating leaves which became inverted on the water surface and were inoculated. The fact that these lesions were slower to develop on the abaxial epidermis may be related to infection court differences between the two floating leaf surfaces. Floating leaf adaxial cell walls were covered by a papillate waxy layer and numerous hairs not present on the abaxial leaf surface. In addition to topographical differences, it may be that the two floating leaf surfaces have different leaf exudates, with those of the adaxial epidermis being more stimulatory. Yadav (1981) suggested that leaf exudates could be important for successful penetration. Confronted by different topographies and possibly quality or quantities of leaf exudates, H. oryzae appeared to have colonized mesophyll cells underlying the adaxial epidermis more extensively at 48 h p.i., than

those underlying the abaxial epidermis (Fig. 18, A,B,G-I).

d. Sporulation

Despite the relatively narrow indicated host range of H. oryzae in temperate North America, it has been implicated as the most severe incitant of brown spot in wild rice (Kernkamp et al., 1976). Since the pathogen has now been shown to be capable of causing progressive infections in floating leaves, its ability to sporulate on such tissue must determine to a large extent the importance of the floating leaves in relation to the severity of brown spot epidemics.

The stages of sporulation are indicated in Fig. 19 and corresponded to those described by Fukatsu and Kakizaki (1955) for rice leaves. On the leaf surface emergent conidia appeared grey initially, but brown pigmentation developed as they matured. These mature conidia and their associated conidiophores are shown in Fig. 19, A. Conidiophores appeared to emerge first from areas on the edges of the lesions, however eventually even necrotic areas supported sporulation (Fig. 19, B-E). At 7-8 days post-senescence, sporulation was independent of lesion location (Fig. 19, F). At this stage, senescent leaves were covered by a greyish-brown film. The collapsed nature of the senescent leaves precluded plastic-embedding, so that emergence through stomata, observed by Fukatsu and Kakizaki (1955) could not be confirmed. However, the swollen nature of infected host epidermal cell walls may be difficult for the pathogen to penetrate and wild rice stomates would provide a natural opening.

The relationship between leaf senescence and sporulation could be due to nutrient deficiencies as sporulation of H. oryzae occurred when the mycelium had exhaused its nutrient supply (Matsuura, 1973).

Fig. 19. Sporulation of Helminthosporium oryzae on infected wild rice floating leaves.

A) Conidiophores and conidia of Helminthosporium oryzae. x 200.

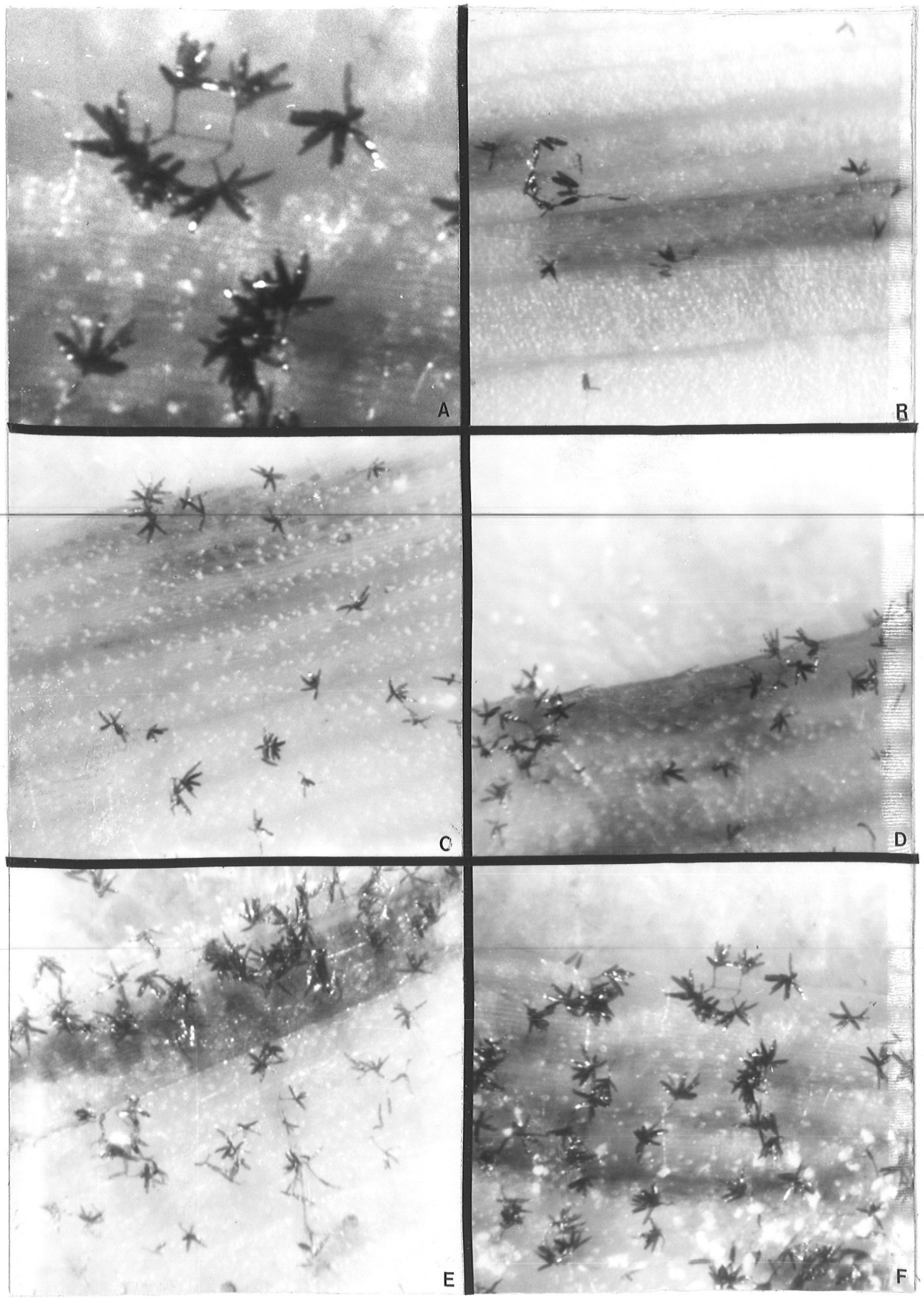
B) Onset of sporulation at the periphery of a lesion. Remaining host tissue already senescent and totally chlorotic. x 100.

C) Sporulation spreading to area between small lesions. x 100.

D) Sporulation on necrotic host tissue of coalesced lesions. x 100.

E) Sporulation at 5-6 days post-senescence on necrotic areas and intervening host tissue. x 100.

F) Sporulation at 7-8 days post-senescence is generally uniform over the leaf surface and independent of lesion location. x 100.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This may also explain why infected, non-senescent attached leaves of both types failed to support sporulation of the pathogen. Sporulation on floating leaves which have been artificially inoculated was abundant. Under more natural conditions, with a lower concentration of inoculum, floating leaves developed only a few lesions (under 15) per leaf, however sporulation was still abundant on the fewer lesions given that the leaves were senescent. Thus abundant sporulation of H. oryzae from lesioned areas of infected wild rice floating leaves occurred if the leaves were undergoing senescence or detached in some way from the plant. The implications of this abundant sporulation in the Helminthosporium blight disease cycle are discussed in the General Discussion.

D. OTHER INTERACTIONS

The success of Calcofluor in conjunction with epifluorescence microscopy as a technique for studying Helminthosporium oryzae on leaf surfaces suggested that the same procedure might be extended to other interactions. The results of this portion of the study are presented in Fig. 20.

1. Host-pathogen-pollen interactions

a. Observations and evaluation of techniques

Ordinarily, the conidium germinated and produced a germ tube (Fig. 20, A), and pollen grains were deposited in clusters on the aerial leaf surface (Fig. 20, B). Where interactions between the two occurred, three patterns of association were noted: (1) neither pollen grain nor fungal structures was altered (Fig. 20 C,D); (2) pollen grains appeared damaged by the association (Fig. 20, E,G); and (3) the associations between pollen grains and germ tubes of H. oryzae were adjacent to lesions. These lesions were characterized by host cells which either autofluoresced bright yellow (Fig. 20, E,F) or which exhibited dark brown pigmentation (Fig. 20, H-J). Brown pigmented host cells were also found on occasion underlying pollen in the absence of the pathogen, suggesting that pollen itself might injure host cells thereby rendering them more susceptible to attack. Another possibility was that the presence of pollen grains speeded up the process of lesion formation by contributing to the inoculum potential of the pathogen. These conclusions are supported by the observation that large brown lesions associated with pollen-pathogen interactions appeared approximately 12 h earlier than similar lesions initiated on aerial leaves by H. oryzae alone.

Fig. 20. Interactions between Helminthosporium oryzae, pollen grains, and cyanophyte algae on wild rice leaves.

Host-pathogen-pollen - 22 hours post-inoculation (A-H)

A) H. oryzae conidium germinating on the aerial leaf in the absence of pollen (C;Fl-I). x 280.

B) Autofluorescent thin-walled pollen grains of wild rice (-;F3-II). x 532.

C) Germ tubes of one germinating spore in association with two pollen grains (C;F3-I). x 333.

D) Most frequently observed pattern of association involving one or two germ tubes in contact with a large cluster of pollen grains (C;Fl-I). x 135.

E) Pollen-associated conidium inducing typical yellow autofluorescence of infected host cells. The fluorescent yellow and collapsed appearance of the pollen grain may indicate damage (C;Fl-I). x 405.

F) As E), but tungsten corrected film was used. x 135.

G) Collapsed appearance and granular contents of pollen grains may indicate damage as a result of association with germ tubes (C;F3-I). x 200.

H) Small brown lesion underlying two pollen grains (C;Fl-I). x 220.

24 hours post-inoculation (I)

I) Medium-sized lesion in association with a pollen-pathogen interaction (C;F3-I). x 333.

28 hours post-inoculation (J)

J) Large brownish-yellow lesion underlying a pollen-pathogen interaction. Hyphae appear to be penetrating a stomate (C;Fl-I). x 200.

Host-pathogen-algae - 48 hours post-inoculation (K-L)

K) Cyanophyte algae on the abaxial surface of the floating leaf are similar in size and shape to the wild rice pollen grains (C;Fl-I). x 500.

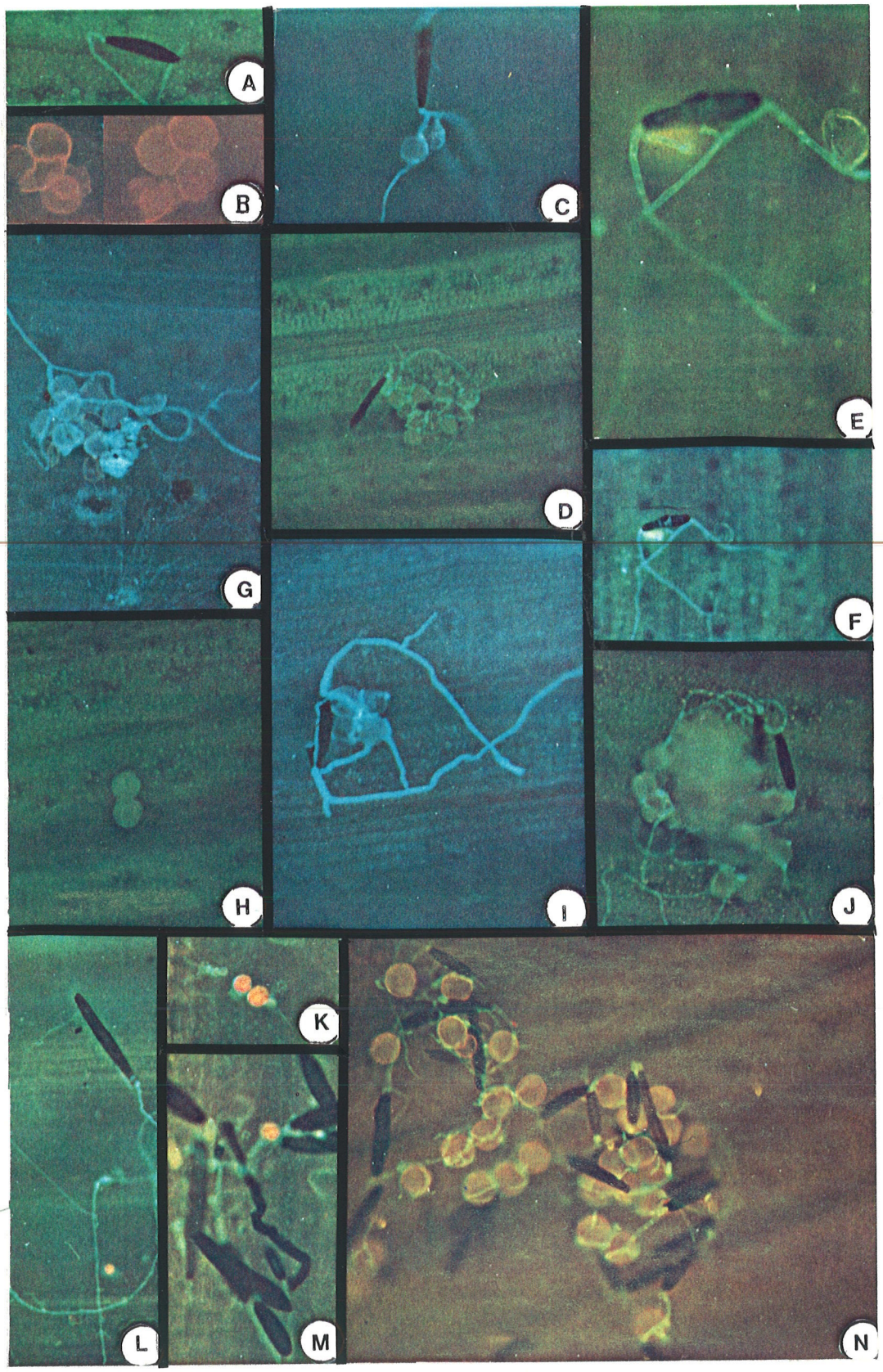
L) No lesions or leaf damage are associated with germ tubes in contact with cyanophyte algae (C;Fl-I). x 260.

Patterns of interactions (M-N)

M) The algae-pathogen association involving random contact between the germ tube and the algal cell (C;Fl-I). x 500.

N) Pathogen interacts with pollen grains by enclosing them in a 4-square network of hyphae (C;Fl-I)*. x 333.

*no blue filter in camera attachment.


A
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Calcofluor facilitiated the visualization and qualitative analysis of the interactions. The Calcofluor-treated germ tubes of H. oryzae fluoresced brilliantly blue, while pollen grains showed a slight natural fluorescence but did not bind Calcofluor. The wild rice aerial leaf surface was also non-fluorescent and host cells were clearly visible. Conidia of H. oryzae, although occasionally showing some Calcofluor fluorescence, generally appeared dark brown and septa were observed. Generally filter system F1-I was adequate for observations, however, F3-I did allow a stronger fluorescence of Calcofluor-treated hyphal walls. Using this filter system, the pollen grains appeared pinkish-white, while with F1-I, they appeared white or yellowish-orange. Also if the blue filter was removed from the camera attachment, the entire system appeared to have a yellowish tint (Fig. 20, N).

Some other attempts have been made to examine pollen-pathogen interactions microscopically. Ogawa and English (1960) presented photomicrographs of almond pollen grains stimulating Botrytis spore germination and germ tube growth, but no details of the method were indicated. Chou and Preece (1968) observed B. cinerea interacting with strawberry pollen in spore germination tests. They also fixed lesioned areas in formal-acet-alcohol, stained them with cotton blue in lactophenol and examined them microscopically. Fokkema (1971) examined epidermal strips of rye leaves infected with H. sativum in the presence of pollen, using Periodic Acid Schiff (P.A.S.). In counting the number of small lesions on P.A.S.-stained leaf pieces, however, Fokkema questioned whether all the lesions counted were in fact sites of penetration because on pollen-free leaves it was sometimes impossible to detect mycelium near or on lesions; mycelium was always detected near lesions on leaves with pollen

because of the high mycelial density. No differential staining between pollen and pathogen was mentioned in any of these papers and was not obvious in photomicrographs.

Epifluorescence microscopy of this pollen-pathogen interaction, taking advantage of the autofluorescence of infected host cells and the preferential uptake of Calcofluor by hyphal walls, has yielded superior results. It seems likely that the use of Calcofluor and epifluorescence microscopy would alleviate the problem encountered by Fokkema (1971) of discerning infected cells and sites of penetration, while at the same time discriminating between pollen associated and pathogen associated lesion types. Superficial mycelial growth would also be easily observable using these techniques.

b. Role in disease incidence

The suggestion by Gilbert (1974), and others, based on greenhouse experiments at the University of Manitoba, that natural pollen deposits on wild rice aerial leaves increase the onset and severity of infection by H. oryzae is supported by observations that epidemics of Helminthosporium blight usually occur when the plants are in flower (Kernkamp et al., 1976). Pollen collected on the leaves of greenhouse grown wild rice plants and was not easily washed off. The flag leaves, which were especially heavily coated, succumbed more quickly to infection, although this was not quantified.

A similar pollen-related increase in disease severity has been reported for other combinations by Ogawa and English (1960), Bachelder and Orton (1963), Chou and Preece (1968), Mansfield and Deverall (1971), and especially Fokkema (1971). The latter found that: (1) substances responsible for the stimulation of H. sativum were present in rye pollen;

(2) the consequent enhancement of growth could lead to an increase in the number of infection sites, which in turn would lead to necrosis; and (3) rye pollen induces the production of cell wall degrading enzymes by H. sativum. One difference between his results and those presented here is that he observed no pollen-associated host tissue discoloration such as is shown in Fig. 20, H. However, it should be noted that natural pollen deposits may have effects which differ from those observed with pollen suspensions and that epifluorescence microscopy allows better differentiation of affected host cells.

The role of pollen in increasing the severity of disease symptoms may be as: (1) a stimulant to the pathogen; (2) a factor in predisposing the host to infection; and/or (3) an incitant of host cell damage. Dr. T. Booth (personal communication) has suggested a combination of structural, nutritional, or chemical factors may explain these roles. Structurally, there may be an affinity between germ tubes of the pathogen and the round shape of the pollen. It may also be that the pollen provides support for more extensive germ tube growth.

Wild rice pollen diffusates may stimulate or nourish the developing pathogen germ tube. Chou and Preece (1968) suggested that sugars in the pollen diffusate were not solely responsible for the stimulatory effect. However, Warren (1972a; 1972b) found that only a mixture of sugars with boric acid reproduced the stimulatory effect. Choline and betaine from wheat pollen extracts are known to stimulate fungal growth (Strange et al., 1974). Similar stimulatory factors may occur in wild rice pollen. H. oryzae leachates, in turn, may prolong the longevity of wild rice pollen grains. Such leachates have been obtained from germinating spores of other Helminthosporium spp. (Yadav and Mandahar, 1981).

Finally, there may be stimulation of wall degrading enzymes by the pathogen or the host induced by the pollen. Barash et al. (1964) noted that leachates from safflower blossoms contained pectic substances that induced the production of polygalacturonase by B. cinerea. Fokkema (1971) suggested that the induction of cellulase and polygalacturonase in H. sativum by rye pollen (host material) before the leaf is penetrated and consequently before the defence mechanism of the host has fully started, may be of great advantage to the pathogen. It may be that wild rice pollen induces these wall degrading enzymes in H. oryzae conidia, or indeed in host cells, resulting in browning. Although the combination of structural, nutritional and chemical factors may contribute to the observed increase in disease severity, identification of the pollen stimulatory factors and research into the quantitative aspects of the host-pathogen-pollen interaction must be undertaken before further conclusions can be drawn.

2. Cyanophyte algae-pathogen interaction

The interaction between cyanophyte algae and H. oryzae germ tubes is illustrated in Fig. 20, K-M.

The technique used with the pollen-pathogen interaction was equally effective in discerning the algae-pathogen interaction. Fungal germ tubes fluoresced brilliantly blue when treated with Calcofluor. The algal cell walls bound Calcofluor to a limited extent, but were readily distinguished by the strong red fluorescence imparted by chlorophyll 'a' (Dr. S. Badour, personal communication). Damaged host cells which were either yellow autofluorescent or brown pigmented were not observed in relation to the alga-pathogen interaction or algae separately. This indicated that there was no apparent host damage as a result of algal

presence.

Calcofluor has been used to fluoresce the walls of marine algae (Cole, 1964), but not to assess the role of algae in host-pathogen interactions. This technique could be used to assess the damage, if any, caused to floating leaves by algae blooms in the field.

No role in increasing or decreasing disease incidence under greenhouse or growth chamber conditions could be attributed to cyanophyte algae. Their presence, unlike that of pollen, does not appear to affect the symptoms resulting from the host-pathogen interaction on the floating leaves.

3. The pattern of the interactions

The nature of the pollen-pathogen interaction appeared to be one of a definite pattern of hyphal growth around each pollen grain in a 4-square conformation (Fig. 20, N). This type of pattern has been found on fossil pollen which has been in contact with fungal hyphae (Dr. T. Booth, personal communication). Fungal parasitism of pollen is an extremely common phenomenon and fungi apparently find nutrients in the cytoplasm and pollenkitt surrounding the grains (Vasanthy and Pocock, 1981). Fig. 20, E-G illustrates damaged pollen which may have been parasitized, however such damage was rarely observed. It appears, then, that the 4-square conformational pattern around the pollen grains may allow the germ tubes access to leached nutrients or provides a structural relationship, while leaving the pollen grains visibly intact.

The pattern of the interaction between pathogen germ tubes and algal cells had no such specificity (Fig. 20,M). Algal cells remained undamaged by the encounter, showing no signs of having been parasitized, however, there was contact between the pathogen cell walls and algal cells.

Germ tubes often germinated in the direction of algal cells on the floating leaf surface.

The specific nature of the two interactions may also reflect the results previously observed in relation to lesion association. Pollen represents host material and may be identifiable to the pathogen on a chemical basis in a manner similar to that of leaf cells. Algae, on the other hand, would not be expected to relate to the pathogen in this way.

E. GENERAL DISCUSSION

A general evaluation of all the microscopy and microtechniques which were found to be satisfactory for use with healthy aerial and floating leaves of wild rice and those infected with Helminthosporium oryzae is given in Appendix IV. This study introduced the use of epifluorescence microscopy for wild rice-pathogen-pollen interactions and constituted the first use of fluorochromes Calcofluor and ethidium bromide (EB) to discern most H. oryzae infection structures. However, hyphae of H. oryzae within aerial leaf lesions did not fluoresce when treated with Calcofluor or EB, as expected, based on studies with other fungal pathogens (Rohringer et al., 1977; Kuck et al., 1981). Patton and Johnson (1970) working with Cronartium ribicola J. C. Fisher ex Rabenh observed that while germ tubes on the surface were brightly fluorescent, at the point where the germ tube passed between the guard cells, the fluorescence imparted by Calcofluor was quenched, and that none of the subsequently formed infection structures (substomatal vesicle, infection hyphae) showed any blue fluorescence. They did observe, however, that these structures could be seen as faintly autofluorescent. The quenching of Calcofluor fluorescence was thought to indicate the point of interaction between the host and the pathogen. Rohringer et al. (1977) suggested that the reason the Calcofluor technique proved unsatisfactory for use with Pyrenophora trichostoma (Fr.) Fckl. was that the fungus-associated melanin in affected cells absorbed much of the light required for fluorescent excitation. In working with stem rust of wheat, Kuck et al. (1980) suggested that haustoria in necrotic cells did not fluoresce because

of the conditions in these cells which may have sealed off the haustoria and prevented staining. It may be that compounds in affect-wild rice cells prevented either the visualization of Calcofluor-fluorescent hyphae or a reaction of the hyphae with Calcofluor. It may also be that invading H. oryzae hyphae have a different wall composition from the superficial germ tubes and appressoria, and hence do not fluoresce similarly. Although Calcofluor-treated hyphae were not visible within aerial leaf lesioned areas, they were quite prominent within whole mounts of brown-ringed floating leaf lesions (Fig. 18, C-F). Walls and silica of floating leaf epidermal cells may not scatter the UV illumination as much as their aerial leaf counterparts. This would allow for enhanced excitation of the fluor thus rendering the fluorescent hyphae more visible. It would also account for the brighter yellow autofluorescence observed in affected floating leaf cells as compared to those of aerial leaves. Most other fluorochromes tested in this study (Appendix II) proved unsatisfactory for pathogen differentiation or for use with healthy tissue, except aniline blue (AB), which differentiated healthy host cells, and like Coriphosphene O, did cause yellow-green fluorescence of the pollen grains.

One major problem encountered in obtaining plastic-embedded thin sections of either infected or healthy leaf tissue was the fluctuating quality of the JB-4 plastic. Since plastic- or resin-embedded thin sections have been shown to give a much finer resolution of detail than either free-hand or paraffin-embedded sections (Feder and O'Brien, 1968; Warmke and Lee, 1976; Bennett et al., 1976), glycol methacrylate (GMA) was recommended for use with plant tissue. GMA (Appendix I), however, also proved unpredictable in that bubbling occurred around

lesioned areas during the GMA embedding process, a phenomenon also noted by Smith (1982). Although the use of Spurr's resin (Appendix III) for thin, as well as ultra-thin sections could alleviate these probelms, it might possibly limit the use of stains and fluorochromes. Another problem unlikely to be solved by using an alternate plastic is that the high silica content of the floating leaves made sectioning using glass or diamond knives extremely difficult. Although Smith (1982) found methylene blue (MB) inadequate for GMA-embedded thin sections, its use in this study allowed a correlation between whole mounts and thin sections with respect to the build up of phenolics during lesion formation, and also adequately differentiated fungal structures. None of the other conventional stains tested allowed this type of correlation and often did not even adequately differentiate the fungal structures.

Electron microscopy procedure 5 (Appendix III), although satisfactory for use with healthy aerial leaf tissue, caused problems with floating leaf tissue which lay not in the procedure per se, but rather in the hydrophobic nature of these leaves. In order to fix them in an aqueous fixative, it was necessary to reduce the pressure to remove the air from them and to repeat this procedure frequently during fixation. Floating leaves also seemed less amenable to washing with buffer. However, once the dehydration and infiltration were under way, water was longer the solvent and the problem was eliminated. It may be necessary, however, to modify this procedure for use in future host-pathogen studies and controls must be rigorously compared with their healthy counterparts.

This study provided new insights into the infection process of

Helminthosporium oryzae on aerial and floating leaves of wild rice. The minor differences in pathogen behavior reflected differences in developmental physiology and epidermal morphology of these leaf types. Time factor differences between the two interactions appeared related to the infection court presented to the pathogen. Walls of floating leaf epidermal cells were thinner, more papillate, and covered by a waxy layer, and the leaf surface had numerous prickle hairs. In addition to these physical obstacles, not encountered on the aerial leaves, the pathogen, in nature, enters the infection court under sub-optimal spring temperatures. Assuming that leaf exudates are important for successful penetration of the host (Purkayastha and Mukhopadhyay, 1974; Yadav, 1981) those of the floating leaves were more likely to be removed by washing and may also have been less stimulatory to H. oryzae. The aerial leaf infection court with its associated pollen deposit and leaf exudates may have stimulated better and faster pathogen penetration. Differences in pathogen behavior towards to the two leaf types were also reflected in the sporulation studies (Table 1). Given the same leaf area, H. oryzae produced twice as many conidia on aerial leaves as on floating leaves and this may indicate differences in leaf chemistry between the two leaf types. Despite these minor differences in behavior, however, floating leaves represent a point in the life cycle of wild rice at which H. oryzae can establish itself on the host and subsequently produce abundant conidia which when air-borne to the aerial leaves quickly cause destructive lesions. Clearly, then, infected floating leaves are potentially important as a source of inoculum during the reproductive stage of the host life cycle.

Although Helminthosporium oryzae blight has been a major cause of production losses for wild rice growers, little headway has been made in developing resistant selections. A major deterrent has been difficulty growing such selections to the aerial leaf stage under controlled conditions for screening purposes. This study suggests that floating leaves, though quantitatively somewhat different, may be qualitatively similar enough to aerial leaves that they could be used for this purpose. This would greatly reduce the time and cost involved in operating a pathogenicity screening program.

H. oryzae is one of a number of foliar pathogens which attack both Zizania aquatica and Oryza sativa. This may be explained in part by the similar leaf surface environments of these two host plants. Pathologists studying the epidemiology and control of this and other pathogens of wild rice should be thoroughly familar with the rice literature and vice versa.

Further studies suggested by this research include an ultrastructural examination of the Z. aquatica-H. oryzae interaction and other wild rice-pathogen interactions; comparisons of the behavior of other pathogens on floating versus aerial leaves; evaluation of systems for pathogenicity testing based on floating and aerial leaves; and investigations, both qualitative and quantitative, of host-pathogen-pollen interactions.

SUMMARY

1. Healthy wild rice aerial leaves showed typical gramineous features and had many features in common with other Oryzeae members, in particular Oryza sativa and Leersia spp., which are alternative hosts of the wild rice pathogen Helminthosporium oryzae.
2. Infection of aerial leaves by H. oryzae involved direct and stomatal penetration. The pathogen progressed intracellularly in penetrated epidermal cells, vascular bundles, and bundle sheath cells, and intercellularly in areas of mesophyll cells.
3. Infected aerial leaf cells showed a bright yellow autofluorescence, and as lesions developed, the autofluorescent host tissue changed color from bright yellow to orange. As final lesion color was achieved, autofluorescence was quenched by brown pigments.
4. Three lesion types developed on the aerial leaves and these were designated as coalesced, haloed, and discrete.
5. At the cellular level, the pathogen destroyed mesophyll cell walls and organelles, however un-invaded epidermal cells appeared to remain intact, with some conformational wall changes and swelling.
6. Sporulation on aerial leaves did not occur while the leaves were still green, despite heavy infection.
7. Healthy wild rice floating leaves exhibited many internal features similar to those of aerial leaves. However, there were two distinct floating leaf epidermal surfaces, which differed again from the uniform nature of the aerial leaf epidermis.
8. Infection of floating leaves by H. oryzae involved similar pre-infectional stages, however, the duration of these stages was longer.

9. The bright yellow autofluorescence of infected floating leaf cells appeared brighter than that of their aerial leaf counterparts and quenching of autofluorescence as brown pigmentation increased occurred in a more gradual manner in floating leaves.
10. Four lesion types developed on the floating leaves which were designated discrete, haloed discrete, brown-ringed and coalesced.
11. Sporulation of the pathogen on the floating leaves was observed only if senescence of attached diseased leaves had occurred or if non-senescent diseased leaves were artificially detached from the plant.
12. Infected floating leaves appeared to be capable of providing a potential source of H. oryzae inoculum.
13. Wild rice pollen on aerial leaf surfaces interacted with H. oryzae germ tubes. This interaction was often lesion-asociated which indicated that the presence of pollen on the leaves may increase the severity of the infection by the pathogen.
14. Cyanophyte algae on the floating leaves were found in association with H. oryzae germ tubes, but these were not lesion associated, and the nature of the interaction differed from the definite pattern of hyphae on the pollen grains.
15. Fluorescence and light microscopy of cleared leaf segments treated with Calcofluor, ethidium bromide (EB), and aniline blue (AB), and of thin JB-4 plastic-embedded sections stained with acid fuchsin/ toluidine blue (AF/TB), TB alone, or methylene blue (MB) were satisfactory for both aerial and floating leaves. Techniques were developed for the examination of ultra-thin sections and could be modified, if necessary, for use with infected host tissue.

16. Differentiation of H. oryzae from affected and unaffected host cells was achieved by treating mounts with Calcofluor or EB or by staining thin sections with MB. Infected host cells were distinguished by yellow autofluorescence or bluish-green color with MB. Calcofluor-fluorescent germ tubes were easily distinguishable from non-autofluorescent wild rice pollen grains and red fluorescent cyanophyte algae with which they were interacting.
17. The infection studies indicated that floating leaves may provide plant breeders with a cheaper, faster system for initial screening of wild rice varieties for resistance to H. oryzae.

APPENDIX I.

Glycol Methacrylate Embedding Procedures

Glycol methacrylate mix:

GMA (2-Hydroxyethyl Methacrylate)	93%	46.5 g
Carbowax 200	7%	3.5 g
Benzoyl peroxide	0.2%	0.1012 g

Infiltration:

Tissues are transferred to 50% butanol/ 50% GMA mix (above) from 100% butanol, and vials are placed on a rotor at room temperature. Tissues are left in the 50/50 mixture for 24 hours with one change. This mixture is replaced with 100% GMA mix (approximately 3-4 mls just covering the specimens), and vials are left on the rotor overnight. Specimens are then placed in the cold ($4^{o}C$) for one month with 2 changes of 100% GMA mix.

Embedding

Fresh GMA mix is prepared for the embedding procedure. Specimens are placed in foil dishes and covered with approximately 8.5 mls of GMA mix. Another foil dish is placed on top to dispel the air to ensure proper polymerization. The foil dishes are placed in a $60^{o}C$ oven overnight (17 hours). Foil dishes are allowed to cool and then the foil is removed and the block is prepared for sectioning.

APPENDIX II.

Evaluation of the Use of Fluorochromes

Fluorochrome	Healthy leaf tissue	H. oryzae	Lesions	Pathogen Interactions
Autofluorescence	++	-	++	++
Aniline Blue	++	-	/	/
Ethidium bromide	++	++	++	/
Calcofluor	++	++	/	++
Coriphosphene O	+	+	/	/
Nile Blue A	+	-	/	/
Primuline	+	-	/	/
Congo Red	+	-	/	/
Rhodamine B	-	-	/	/
Auramine O	-	+	/	/
Acriflavine	-	+	/	/
Picridine Orange	-	+	/	/

(/) Fluorochrome was not applied
(++, +, -) Fluorochrome was applied

(++) - fluorochromes gives excellent differentiation satisfactory for photomicrography
(+) - differentiation was observed, but not satisfactory for photomicrography
(-) - no differentiation, therefore no photomicrography was done

Appendix III

Schedule of electron microscopy procedures

Summary of Electron Microscopy Procedures

Steps:	Procedure 1	2	3	4	5
Fixation #1	1.5% glut. in 0.025M phosphate buffer, pH 6.8, 0.75% acrolein no vacuum, for 2 hr.		as #1/2 but no acrolein; vacuum 26 lb. for 1 hour		
Fixation #2	6% glut. in 0.025M phosphate buffer, pH 6.8, overnight		as #1	as #1	as #1
Buffer washes	0.025M phosphate buffer 4 x 15 min.		9 x in 48 hours	as #3	as #3
OsO_4 (4% in 0.05M Phosphate buffer)	1 1/2 hr.	as #1	12 hr in ice	as #3	as #3
H_2O washes	4 x 15 min.	as #1	3 x 20 min.	as #3	as #3
Ethanol	30,50,70,90 x 30 min each 100% x 3 x 15 min each		20,40,60,80,95 x 30 min ea. 100% x 3 x 15 min. each		10,20,30,50,70 90,95. 100%,100% 100% x 30 min.ea.
Ethanol/ Propylene Oxide	not used	3:1;1:1;1:3 100% x 3x 1 hr	as #2	not used	9:1;4:1;3:1;1:1; 1:3;100%;100%;100% 1 hr. each
Spurr Infiltration	EtOH/Spurr 3:1;1:1;1:3; 100%,100%,100% 1 hr each/overnight	P.O./Spurr 3:1;1:1;1:3 100%,100%,100% 1 hr each/overnight	as #2	as #1	as #2
Spurr Changes	100% once per day for 3 days		as #1	as #1	as #1
Spurr Embedding	60°C/15 lb. overnight	60°C overnight no pressure	as #2	as #1	as #2

APPENDIX IV

RECOMMENDATIONS FOR USE OF MICROSCOPY AND MICROTECHNIQUES

Epifluorescence microscopy

Whole leaf clearing:
- -use with Calcofluor, aniline blue, ethidium bromide on both leaf types for general healthy tissue staining
- -infected host cells autofloresce yellow consistently
- -pathogen H. oryzae infection structures fluoresce well with Calcofluor and ethidium bromide

Sectioned tissue:
- -Calcofluor and aniline blue can be used on uninfected host tissue
- -autofluorescence of infected host cells
- -pathogen treated with Calcofluor fluoresced in floating leaf brown-ringed lesions only

These techniques can be used equally effectively on both leaf types

Conventional light microscopy

JB-4 plastic embedded sectioned tissue:
- -use with acid fuchsin, toluidine blue, methylene blue on both leaf types for general healthy tissue staining
- -in infected tissue the pathogen is well differentiated from infected host cells and healthy host cells using methylene blue (1% in 1% sodium borate); pathogen (purplish) and infected host cells (bluish-green).

Whole leaf clearing:
- -toluidine blue, trypan blue, cotton blue can be used to identify infection structures of the pathogen, but differentiation is greater using epifluorescence microscopy.

Glycol methacrylate embedded sectioned tissue:
- -use only with healthy aerial or floating leaf material, however differentiation is not as good as with JB-4 plastic
- -infected host tissue do not embed well using this plastic and bubbling results around lesioned areas

These techniques work best with aerial leaves. Floating leaves can be prepared for examination using conventional light microscopy however the silica content of the floating leaves makes sectioning extremely difficult.

Electron microscopy

- -procedure 5 (Appendix II) is excellent for aerial leaves
- -some problems occur with floating leaves using procedure 5, however, extending the scheduleis not recommended as the extension may damage the cells at the ultrastructure level. The problem apparently arises from the hydrophobic nature of the epidermis of the floating leaves.

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