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THE UNIVERSITY OF ALBERTA

NATURE OF RESISTANCE AND SUSCEPTIBILITY IN TWO NEAR-ISOGENIC VARIETIES OF FLAX TO THE RUST FUNGUS *MELAMPSORA LINI* (PERS.) LEV.

by

MOHAMED RAAFAT EL-GEWELY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "NATURE OF RESISTANCE AND SUSCEPTIBILITY IN TWO NEAR-ISOGENIC VARIETIES OF FLAX TO THE RUST FUNGUS *MELAMPSORA LINI* (PERS.) LEV." submitted by Mohamed Raafat El-Gewely in partial fulfilment for the requirements of the degree of Doctor of Philosophy.

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Date

March, 19, 1971.

ABSTRACT

Two near-isogenic varieties of flax, differing in their reaction to *Melampsora lini* (Pers.) Lev. f.sp. *liniperda* Koern race '300' were involved in this study to elaborate on the nature of resistance. The reaction to the rust fungus in these near-isogenic varieties is determined by a monogenetic factor.

Cytological studies using light and electron microscopy showed differences between inoculated cotyledons of the susceptible (Redwing) and immune (S-66-53) flax varieties. In Redwing the fungus produced extensive mycelium and prominent haustoria. However, in S-66-53 no haustoria were formed, but a hypersensitive reaction was observed. The fungus did not appear to establish any nutritional relationship in S-66-53.

Antigens of Redwing and S-66-53 were found to be similar using the double diffusion technique in agar gel.

Total protein decreased with age in non-inoculated cotyledons of both flax varieties. Non-inoculated and inoculated cotyledons of Redwing and S-66-53 contained similar levels of protein concentration. The number and the patterns of protein bands as determined by disc gel electrophoresis did not differ in these near-isogenic varieties before or after inoculation.

Phenol-oxidizing enzymes, i.e. peroxidase and polyphenol oxidase(s) were studied. Peroxidase activity increased significantly in both varieties with age of the cotyledons. However, peroxidase activity or the number of isoenzymes of non-inoculated or inoculated cotyledons did not appear to be involved in resistance or susceptibility in these

flax varieties. Polyphenol oxidase(s) activity was generally low, though polyphenol oxidase isoenzymes were observed in electrophoretic gels. This enzyme also did not appear to be related to the type of resistance controlled by the R-locus.

The results of β-glucosidase and esterase(s) which can release phenol moieties from larger compounds did not seem to have any role in disease resistance or susceptibility operating in the above near-isogenic varieties.

Catalase, another oxidative enzyme, was studied and its activity in healthy cotyledons was found to be much higher in Redwing than in S-66-53. Non-inoculated cotyledons of an F_1 hybrid (immune) between these two near-isogenic varieties was found to have less catalase than Redwing. Catalase activity was found to decrease in a sequential manner from the young to the old tissue. Extracts of older cotyledons, whether inoculated or not, did not show any detectable catalase activity.

The relationship of the above studies to the nature of resistance in these near-isogenic varieties are discussed and a hypothesis is suggested to explain the genetic control of resistance before and after inoculation as controlled by the R-locus.

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INTRODUCTION

Disease resistance is a general characteristic when the number of pathogens which attack plants are considered. There are about 7,000 - 10,000 species of fungi, 200 bacterial species, and some 300 viruses known to cause disease in plants (Bollard and Mathews 1966). No more than about 153 species of some 50,000 species of fungi attack one single crop (Yarwood 1967). McNew (1960) concluded that parasites are in a constant process of evolution in which they progress from non-parasitic associative relations with the host through facultative saprophytism and parasitism to obligate parasitism. Simultaneously, there is a reduction in the tendency to destroy host cells by secreting destructive substances and the pathogen tends to become associated with the host cells in a compatible arrangement. However, the concept of obligate parasitism is in question since certain species of rust fungi have been grown saprophytically in culture (Scott and Maclean 1969) including *M. lini* (Turel 1969, a and b).

The nature of resistant characters, aside from their mode of inheritance, has been a subject of some interest since about 1890 (Walker and Stahmann 1955). Biffen (1905) was the first to apply Mendel's laws of inheritance,soon after their rediscovery in 1900, in his studies concerning inheritance of disease resistance. Freeman (1911) concluded that the heritable factors were in many cases not reflected in visible properties of structures of the resistant plant. The first study on the inheritance of flax resistance to the rust fungus, *M. lini*, was made by Henry (1930). Parasitic specificity of the flax-rust fungus (*M. lini*) system was concluded to have its genetic basis in the host and

the pathogen (Flor 1955; 1956). Flor's extensive studies led him to formulate the gene-for-gene hypothesis. This model indicates that for each genetic locus in the host governing resistance and susceptibility, there is a specific and related locus in the parasite that governs its virulence and avirulence. This model appears to be operating in many host-parasite systems (Person 1967). A comprehensive review of genetics of the host-parasite systems was carried out by Day (1966). Person (1967) suggested a co-evolution model which combined genetic polymorphism in the host and in the pathogen.

The nature of resistance in plants to invading pathogens has been subjected to extensive cytological and biochemical studies. Cytological studies using light and electron microscopy to investigate the nature of resistance and host-parasite interface were reviewed (Akai 1959), and/or carried out by several workers (Fraymouth 1956; Green and Dickson 1957; Ehrlich and Ehrlich 1962; 1963; Peyton and Bowen 1963; Berlin and Bowen 1964; Shaw and Manocha 1965; Bracker 1968). Ultrastructure of fungi was recently reviewed by Bracker (1967).

Factors affecting infections and development of *M. lini* on flax were extensively studied by Hart (1926), and the cytology of *M. lini* have been studied by Allen (1934). Manocha and Shaw (1967) using electron microscopy, studied the structure and the development of *M. lini* uredospores germinated on distilled water and on a susceptible flax variety. They indicated the dicaryotic nature of uredospores, intercellular mycelium, and haustoria.

The physiological nature of resistance of plants to microorganisms was reviewed by several workers (Hart 1949; Walker and Stahmann 1955; Allen 1959; Barnett 1959; Müller 1959; Rubin and Artsikhovskaya 1963; Shaw 1963; Tomiyama 1963; Farkas and Solymosy 1965; Kuć 1966; Hare

1966; Goodman *et al.* 1967; Shaw 1967; Wood 1967; Metlitskii and Ozeretskovskaya 1968).

There are two types of disease resistance operating in plants; preformed and post-infection resistance mechanisms. The preformed mechanisms of resistance are those which exist in plants prior to inoculation and are independent of infection. These preformed mechanisms could be attributed to one or more of the following factors, (a) fungitoxic compound which is more fungitoxic than phytotoxic, or it is bound in a nontoxic compound from which a toxic moiety is released after infection, (b) factors which could inactivate substances released by the pathogen, (c) deficiency of substance(s) in the host required for the normal development of the pathogens, and (d) structures in the host which prevent the plant from being infected. The post-infection mechanisms of resistance are those induced only as a result of the host-parasite interaction. These induced factors could be any of the following, (a) hypersensitivity, which encompasses all morphological and histological changes that, when produced by an infectious agent, elicits the premature dieing off (necrosis) of the infected tissue as well as inactivation and localization of the infectious agent, (b) increase in concentrations of compounds potentially fungitoxic (e.g. phenols), (c) *de novo* synthesis of phytoalexins which are fungitoxic phenolic compounds produced only after host-parasite interaction, and (d) structural changes formed after infection to prevent further development of the pathogen.

Specific biochemical examples of resistance have been studied. Protein was found in higher concentration in resistant varieties of cabbage than in susceptible varieties (Rubin and Artsikhovskaya 1963). Inoculation with pathogens was found to change protein quantitatively

and/or qualitatively as determined by gel electrophoresis (Staples and Stahmann 1964;1964; Rudolph 1964). Uritani and Stahmann (1961) concluded that resistance might be correlated with the ability of the host tissues to alter their protein metabolism in response to the infecting agent, and then form protein(s) which cannot be detected or could be found only at a low level before inoculation. Common antigens between hosts and parasites were reported to be present in susceptible hosts more than in the resistant hosts (De Vay *et al.* 1967). Doubly *et al.* (1960 a and b) found that a specific antigen in each of four races of the *M. lini* was commonly shared by only those lines of flax that were susceptible to a particular race. A race was virulent to varieties containing its specific rust antigen as a minor constituent, and avirulent to varieties lacking that antigen.

Phenolic compounds were implicated in disease resistance in many host-parasite systems before and after inoculations with pathogens. Phenolic compounds are widely distributed in plants and second only to carbohydrates in abundance (Hayaishi and Nozaki 1969). They are usually found in a bound form as esters and glycosides (Rubin and Artsikhovskaya 1964; Towers 1964; Pridham 1965). Newton and Anderson (1929) were the first to recognize the importance of phenolic compounds in rust resistance in wheat. The role of phenolics in host-parasite interactions and disease resistance has been the subject of many reviews (Pridham 1960; Farkas and Király 1962; Cruickshank 1963; Cruickshank and Perrin 1964; Rubin and Artsikhovskaya 1964; Rohringer and Samborski 1967; Tomiyama *et al.* 1967; Biehn *et al.* 1968 a and b; Kosuge 1969; Veech 1969). Phenolic compounds were observed to be either present in higher concentrations in resistant varieties than in susceptible varieties (El Naghi

and Linko 1962; El Naghi and Shaw 1966) or they were actively synthesized during inoculation with the pathogen in higher concentration in resistant than in susceptible hosts (Kosuge 1969).

Quinones are formed as a result of phenols oxidation. Quinones are extremely fungitoxic. Their antimicrobial activity is attributed to their reactions with proteins or intracellular amino acids, alteration of the cellular redox potential, interference with cofactors and enzyme synthesis, and inhibition of specific enzyme systems (Hoffman - Ostenhof 1963; Webb 1966).

Peroxidase and polyphenol oxidase(s) were found to oxidize phenols into quinones compounds (Conn 1964; Saunders *et al.* 1964; Kosuge 1969) though their precise role *in vivo* is not well understood (Hayaishi and Nozaki 1969; Scandalios 1969). The possible involvement of these enzymes in disease resistance was recently reviewed (Lovrekovich *et al.* 1968; Kosuge 1969; Jennings *et al.* 1969; Rautela and Payne 1970). The level of peroxidase activity in potato tissues before infection was reported to be positively correlated with resistance to *Phytophthora infestans* (Rubin *et al.* 1947; Kedar 1959; Umaerus 1959; Fehrman and Dimond 1967). Lovrekovich *et al.* (1968), found a positive correlation between the level of peroxidase in tobacco leaves and resistance to *Pseudomonas tabaci*. Inoculations with the pathogen was found to increase the peroxidase activity and/or the number of peroxidase isoenzymes* (Uritani and Stahmann 1961; Loebenstein and Linsey 1963; Rudolph and Stahmann 1964;

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*The term isoenzymes will be used throughout this manuscript to describe the different forms of proteins which exhibit the same enzymatic specificity (Webb 1964).

Tomiyama and Stahmann 1964; Weber and Stahmann 1964; 1966; Clare *et al.* 1966; Maxwell and Bateman 1967; Macko *et al.* 1968; Bates and Chant 1970; Rautela and Payne 1970). The role of peroxidase in cell regulation and its involvement in IAA oxidation was recently reviewed by Galston and Davies (1969).

Andreev and Shaw (1965) observed, in their studies, on a susceptible and a resistant flax varieties to *M. lini*, that infection induced premature appearance of two isoenzymes in the resistant variety that would have appeared at later stages in the non-inoculated cotyledons.

Polyphenol oxidase activity and/or the number of the isoenzymes were found to increase after inoculation with the pathogen (Farkas and Király 1962; Farkas and Lovrekovich 1965; Hyodo and Uritani 1966; Stahmann *et al.* 1966; Hyodo and Uritani 1967; Lovrekovich *et al.* 1967).

Peroxidase and polyphenol oxidase were found to inactivate various biologically active proteins (Sizer 1953; Cory *et al.* 1962; Cory and Frieden 1963). These enzymes were found to be involved in phenol and lignin biosynthesis (Conn 1964; Schubert 1965; Brown 1966; Asada and Masumoto 1967; Hayaishi and Nozaki 1969; Kosuge 1969). The subject of peroxidase and polyphenol oxidase isoenzymes were recently reviewed (Scandalios 1969; Shannon 1969).

Another oxidative enzyme, catalase, was studied in relation to disease resistance and in host-parasite interactions. Maxwell and Bateman (1967) found that catalase activity increased in extracts of *Rhizoctonia*-infected bean hypocotyls. This increase in catalase activity was found to be limited in the necrotic areas *per se*. Rudolph and Stahmann (1964) suggested that the catalase of pathogens may be one factor for virulence in that the enzyme depresses the activity of peroxidase involved in the

defence reaction of the host.

Hydrolytic enzymes such as β-glucosidase and esterase(s) were found to release phenols from phenolic glucosides and esters (Pridham 1963; Conn 1964; Kosuge 1969). Recent reports on the physiological nature of resistance have included studies on β-glucosidase and its possible role in disease resistance (Raa 1968; Hildebrand *et al.* 1969; Powell and Hildebrand 1970). These studies discussed the role of β-glucosidase in releasing phenols from pre-existing host glucosides and subsequent oxidation of these phenols as a defensive mechanism expressed by the resistant host.

Esterase(s) was reported to release phenols from phenolic esters, however, this enzyme has not been studied in relation to disease resistance. Esterase isoenzymes were recently reviewed by Scandalios(1969) and Shannon (1969).

Although disease resistance was found to be inherited, this fact was almost neglected in studies involving nature of resistance mechanisms against invading microorganisms. Since Flor (1956) indicated the importance of using isogenic lines in such studies, very few reports followed this approach.

Host-parasite interface studies were made recently using isogenic or genetically related hosts (Hilu 1965; Van Dyke and Hooker 1969; Stavely *et al.* 1969). Near-isogenic or genetically related hosts differed only in their reaction to the invading pathogens were used in physiological and biochemical studies on the nature of resistance (Zscheile and Murray 1957; Antonelli and Daly 1966; Hilty and Schmitthenner 1966; Stavely and Hanson 1967; Daly *et al.* 1970; Seevers and Daly 1970 a and b).

The objective of the present investigation was to elaborate on

the actual resistance mechanism(s) by studying systems in which the genetic variability was minimal between hosts that reacted differently to the pathogen. Two near-isogenic varieties of flax *Linum usitatissimum* L. were used, and they differed only in their reaction to the race '300' of *M. lini*. These near-isogenic varieties, Redwing (susceptible) and S-66-53 (immune) differed in a monogenetic factor which controls their reaction to the rust fungus. The immune phenotype is the expression of the homozygous dominant alleles *RR* as in S-66-53 or the heterozygous condition *Rr* as in the F_1 hybrid.

In the present investigation the following will be presented: (a) host-parasite interface studies using light and electron microscopy, (b) immunochemical properties of antigens of non-inoculated cotyledons of both varieties, (c) qualitative and quantitative studies on protein of non-inoculated cotyledons of various ages as well as of inoculated cotyledons of both Redwing and S-66-53, (d) qualitative and quantitative studies on enzymes that oxidize phenols, i.e. peroxidase and polyphenol oxidase(s) in non-inoculated cotyledons of various ages as well as in inoculated cotyledons, (e) qualitative and quantitative studies on enzymes involved in releasing phenol moiety from phenolic glucosides and esters, i.e. β-glucosidase and esterase(s) and (f) catalase was also included in this study.

MATERIALS AND METHODS

1. Plant Material

Two near- isogenic varieties of flax *Linum usitatissimum* L. were used throughout these investigations. These were the varieties Redwing, which is susceptible to the flax rust fungus *Melampsora lini* (Pers.) Lev., and S-66-53 which is immune to the rust fungus. These two near-isogenic varieties differed in a monogenetic factor* that controls their reaction to the rust fungus; Redwing is homozygous recessive (*rr*) and S-66-53 is homozygous dominant (*RR*) for this locus. S-66-53 was developed by crossing Redwing *L. usitatissimum* with a wild type flax *L. angustifolium*. The hybrid was back-crossed to Redwing four times, and the immune plants were selected. The fourth back-cross progeny were selfed; it showed 3:1 immune to susceptible respectively. Only immune plants that did not show any segregation in the next generation were selected to form S-66-53 variety (Smith 1967, unpublished data).

2. Growing Conditions

Seeds of both varieties were germinated in 17.5 x 13.2 cm plastic boxes containing a mixture of a moist soil, peat moss, and sand (3:2:1), supplemented with 30g of 20% superphosphate (P_2O_5), 25g of hydrated lime and 3g of 33.5% ammonium nitrate per cubic foot of soil mixture. The flax seeds were germinated under controlled conditions in a growth cabinet in which alternating 12 hrs. of light and dark

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* The mapping of this locus was not carried out.

periods were used. During the light period approximately 1700 foot candles of illumination, measured about 6cm above the soil level, were supplied with 8 fluorescent and 4 incandescent lamps. The temperatures during the light and dark periods were maintained at 20°C and 15°C respectively. The relative humidity in the cabinet was maintained at approximately 60%. Plants were watered daily beginning 3 days from seeding, and Hoagland nutrient solutions I and II were used alternatively in conjunction with the watering.

3. Inoculation and Infection

Inoculations were made by brushing uredospores from rust infected plants onto the cotyledons of 9-day-old seedlings (the age of the plants was calculated from the time of seeding; emergence usually occurred 5-6 days after seeding). The inoculated plants were kept in an illuminated incubation cabinet of high humidity. The humidity was maintained at near saturation by creating a water mist using fine water sprays. These plants were kept under the above conditions for 24 hrs. prior to transfer to the greenhouse at the University of Alberta Parkland Farm.

Uredospores of *Melampsora lini* f. sp. *liniperda* Koern race '300', used as inoculum, were maintained on variety Redwing.

4. Cytological Studies

(a) Fixation and Dehydration Procedures. Non-inoculated, 2-, and 9-day rust inoculated cotyledons were cut into small ribbons, about 1 mm wide, and placed into 3.25% glutaraldehyde in 0.75 M phosphate buffer, pH 7.2, for 3 hrs. at 4°C. These ribbons were rinsed with

cold 10% sucrose, in the above buffer for 3 changes of 1 hr. each. Leaf ribbons were post-fixed with 2% O_8O_4 in the above buffer for 3 hrs. at 4°C, and then rinsed with the above sucrose solution for 2 changes of 30 minutes each. The above plant material was then dehydrated by transferring them through a series of ethyl alcohol solutions containing, 30, 50, 70, 90% and absolute ethyl alcohol, respectively for 30 minutes in each alcohol solution. Prior to the embedding procedure the dehydrated ribbons were taken through 2 changes of propylene oxide of 15 minutes each.

(b) Embedding. The plant ribbons which were previously rinsed in propylene oxide were then put into a 1:1 mixture of propylene oxide and araldite and left over night at room temperature. The above ribbons were then embedded in a fresh mixture of 10 ml araldite and 0.1 ml hardener DM P-30* (Dimethyl a mino methyl phenol) and the blocks were then incubated for 48 hrs. at 60°C.

(c) Sectioning and Staining. Sections of the above embedded plant material were prepared using a Reichert ultramicrotome with a glass knife. Some preparations were stained with toluidine blue** and studied under the light microscope. Remaining sections were stained by placing them into uranyl acetate*** and lead citrate**** for 1 hr. and 3.5 minutes, respectively. These stained sections were then studied with a Phillips EM 200 electron microscope.

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* LADD Research Industries Inc. Burlington, Vermont, U.S.A.

** 1% in 1% borox.

*** 2% in water.

**** 2.25% in water.

5. Immunological Studies

(a) Antigen Preparation. Cotyledons of 15-day-old seedlings of Redwing and S-66-53 were harvested, washed, and frozen at -40°C for 24 hrs. This frozen material was ground with mortar and pestle then stored at -40°C for 4-6 hrs. and then forced through a Hughes press. The resulting disrupted cellular plant sample was allowed to thaw at room temperature and then centrifuged at 32,800g for 40 minutes at 2°C. The supernatant fraction was decanted and centrifuged at 32,800g for 20 minutes to ensure the complete precipitation of disrupted cell fragments. The resulting supernatant was decanted and mixed with an equal volume at 0.05M Tris-HCl buffer, pH 7.5 containing 0.02M Mg SO_4 sufficient $(NH_4)_2SO_4$ was added to the buffered supernatant to give a final concentration of 90% saturation at 25°C. This amount of $(NH_4)_2SO_4$ (662 mg/ml) was data taken from data presented by Green and Hughes (1955). This mixture was stirred at 4°C for 3 hrs. and then centrifuged at 32,800g at 2°C for 15 minutes. The $(NH_4)_2SO_4$ -precipitated fraction was dissolved in a small amount of ammonium bicarbonate solution, 0.002M, and then dialyzed at 4°C against the same solution for 3 changes, each at 3 hrs. The dialyzate was frozen in an acetone - dry ice mixture and then freeze-dried. A portion of the freeze-dried material was dissolved in normal saline* solution (0.85%) and used for immunization after the total protein determination had been carried out. Another portion of the freeze-dried material, stored at -40°C, was used as antigen in the forthcoming experiments. The antigens of Redwing and S-66-53 were designated red - Ag and iso - Ag, respectively. Aliquots of antigens contained 300γ protein (as determined by Lowry method) were subjected to disc gel electrophoresis

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* Saline tablets; Oxoid Division of OXO Ltd., London, S.E. 1.

and assayed for protein bands; and, peroxidase,polyphenol oxidase and esterase isoenzymes (the procedures of electrophoresis and the method employed in detecting protein bands and isoenzymes are described in Protein and Enzyme Studies section).

(b) Immunization. Six rabbits of the variety "San Juan", known to be tolerant in immunization experiments, were used. Prior to any injection of the antigen, a blood sample was taken from each rabbit and treated as control. Three animals were injected with an antigen preparation from Redwing and S-66-53, respectively. In order to build up antibody titer, a prolonged program was adopted, similar to that carried out by Herbert (1966) and Weir (1967). In these procedures 0.05 ml of antigen in saline solution prepared as above and containing 3.5 - 3.7 mg of protein was mixed with an equal volume of complete Freund adjuvant* and injected subcutaneously into each animal. The injections were made just near the limbs from the abdominal side. In the fourth week blood samples were taken from each animal to determine the extent of antibody formation. In the following week, animals were injected with antigen-adjuvant mixture to increase antibody formation. However, in the 6th and in the 7th week the amount of Ag/injected was increased from 3.5 - 3.7 mg to about 5.0 mg. A shot constituted as above was given to the animals two weeks from the previous one. 10 ml aliquots of blood were taken in the 10th week to determine the extent of antibody formation. In the following two weeks similar injections were given to each rabbit as above and in the 13th week a similar shot, but was not mixed with adjuvant, was injected. The last week of immunization program

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* Difco Laboratories, Detroit, Michigan, U.S.A.

(i.e. 14th), a 25 ml blood sample was taken from each of the remaining rabbits for antisera preparation. In the process of immunization, one rabbit from each group was removed due to ill health of one rabbit and sudden death of another. The immunization program is summarized in Table 1.

Table 1

The Immunization Program

Number of Weeks	TREATMENT: Injections		Blood Sample Removed (ml)
	Antigen mg protein/animal	Adjuvant (ml/animal)	
0	-	-	10 ml
1,2,3	3.5-3.7 mg in 0.5 ml n. saline	0.5 ml	-
4	-	-	10 ml
5	3.5-3.7 mg in 0.5 ml n. saline	0.5 ml	-
6,7	5.0 mg in 1.0 ml n. saline	1.0 ml	-
8	-	-	-
9	5.0 mg in 1.0 ml n. saline	1.0 ml	-
10	-	-	10 ml
11,12	5.0 mg in 1.0 ml n. saline	1.0 ml	-
13	5.0 mg in 1.0 ml n. saline	1.0 ml	-
14	-	-	25 ml

(c) Antiserum Preparation. Blood samples taken from each rabbit were centrifuged twice at 32,800g for periods of 15 minutes. 1 ml of merthiolate* was added to each supernatant fraction to prevent microbial growth. The supernatant fraction was used as such, unless otherwise indicated, as the source of antibodies. Antibodies of the above antisera were concentrated according to Rohringer and Stahmann (1958) by the addition of 196 mg/ml of solid $(NH_4)_2SO_4$ to aliquots of the antisera. This amount gave a 33% saturation as determined from table presented by Green and Hughes (1955). This mixture was kept at 4°C for 3 hrs. and agitated several times. The above mixture was centrifuged at 32,800g at 2°C for 15 minutes and the supernatant fraction decanted. The precipitate was dissolved in a small amount of buffered saline solution (0.85% saline in 0.05M phosphate buffer pH 7.0). The antiserum against each of Redwing and S-66-53 antigens will be designated as AS - red and AS - iso, respectively.

(d) Antigen Antibody Test. The interaction of normal sera and antisera on agar gel was investigated using the double diffusion method described by Ouchterlony (1969). LKB** equipment was used for these experiments. The agar gel was prepared as follows: 1.2g ionoagar*** and 1g NaCl were added to 80 ml of deionized distilled water and heated in a water bath until the agar was completely dissolved. 20 ml of the merthiolate solution was added to the still warm agar solution. While

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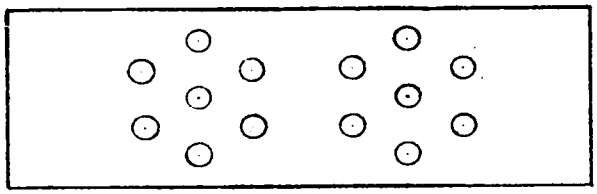
* 0.1% monoethanolamine, Eli Lilly & Co., Canada Ltd. Toronto, Ontario.

** LKB - Produkter AB, P.), Box 12220, Stockholm 12, Sweden.

*** Oxoid Limited, London, England

still warm, sufficient agar solution was poured onto previously cleaned microscopic slides to give a final thickness of about 2mm. After the agar had solidified an LKB well cutter was used to make 3mm diameter well that would hold approximately 5-8λ of either antigen and/or antibody solution. The pattern of wells on each microscopic slide is shown below.



The central well was filled with As - iso, and either red - Ag or iso - Ag respectively, was pipetted in alternate peripheral wells. In another experiment As - red, was added to the central well and iso - Ag and red - Ag, respectively were added to alternate peripheral wells. Also the experiment was repeated using an antiserum of each flax variety in the central well or in the peripheral wells and the corresponding antigens placed into each peripheral well or in the central well respectively. All these preparations were incubated at room temperature (21 ± 1°C) and maintained at high humidity using moist tanks. Observations for precipitation reaction were made daily for 10 days. In the above procedures, if the antigen of one flax variety, is continuous with adjacent arcs resulting from an antigen - antibody reaction of another flax variety, as a result of interaction with an antibody forms an arc, and this arc is continuous with adjacent arcs resulting from an antigen - antibody reaction of another flax variety, both antigenic components would be considered immunologically identical. However, if the arcs were discontinuous,

i.e., either separate or crossed, the antigenic components would be considered immunologically dissimilar.

(e) Immunochemical Absorption Test. To confirm results of the previous antigen - antibody test, and to estimate quantitatively any possible difference in a particular antigenic component, an immunochemical absorption test (Uritani and Stahmann 1961), using the absorption method essentially as described by Wright (1959) was followed.

For this test a film of gelled agar on microscopic slides was used, and agar gel was prepared as described previously. A series of volumetric ratios between As - red diluted with buffered saline (1:1), and red - Ag were prepared. This (1:1) mixture was chosen after a preliminary* immunochemical absorption test was made. The ratios of buffered antiserum to antigen used were 5:1, 4:1, 3:1, 2:1, and 1:1, respectively. The other three combinations As - red ÷ iso - Ag, As - iso ÷ iso - Ag and As - iso ÷ red - Ag, using the above ratios, were also prepared. The mixtures were incubated at 4°C for two days. An aliquot of each antiserum ÷ antigen mixture was placed in each peripheral well and an aliquot of red - Ag or iso - Ag, respectively was placed in the central well. For each of the above combinations, one of the peripheral well was filled with As - red or As - iso. The preparations were incubated at room temperature (21 ± 1°C) in the moist tanks as in the previous test. The development of the reaction was observed daily over a period of 10 days.

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* In this preliminary test equal parts of As - iso and a series of iso - Ag dilution of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively were mixed and incubated as described before.

(f) Photographic Recording of the Immunological Results. Due to the low contrast between the precipitation lines and the agar gel, the procedure described by Hunter (1959) was used for photographic recording of the immunological results. In this procedure, light was directed as nearly as possible normal to the agar surface, without the light source itself being visible to the eye or to the lens. The film used for this study was a Kodak high contrast copy.

6. Protein and Enzyme Studies

(a) Preparation of Extracts. Non-inoculated cotyledons of 9-, 15-, 18-, and 24-day-old seedlings were harvested. Inoculated cotyledons of S-66-53 variety and infected cotyledons of Redwing were harvested 9 days after inoculation (i.e., seedlings were 18 days old). The cotyledons were washed using tap water and then rinsed with deionized distilled water prior to being frozen at -40°C for 24 hrs.

Two preparations were used for assays, one was a crude extract, the other was partially purified using $(NH_4)_2SO_4$.

(1) Crude Extract Preparations: The crude extract was prepared as follows. The frozen samples were ground using a mortar and pestle. The still frozen ground material was then forced through a chilled Hughes press. The resulting disrupted cellular plant sample was allowed to thaw at room temperature, and then centrifuged at 32,800g at 2°C for 40 minutes. The supernatant fraction was removed and centrifuged as above for 15 minutes. The cellular fraction, that precipitates from above centrifugations, were discarded. An equal volume of 0.05M Tris-HCl buffer, pH 7.5 containing 0.02M Mg SO_4 was added to the supernatant. Aliquots of the buffered supernatant were dialyzed at 4°C against the above Tris-

HCl buffer for 3 changes of 3 hrs. each. The dialyzate (crude extract) was used for total protein and enzyme assays.

Another procedure was used for the preparation of crude extracts used for catalase activity studies. In this procedure the fresh plant material was macerated using mortar and pestle at 4°C. The resulting plant slurry was then centrifuged as described above. The supernatant fraction was mixed with aliquots of Tris-HCl buffer and then dialyzed against the same buffer as indicated before.

(2) Partially Purified Protein Fraction: Sufficient solid ammonium sulfate was added to aliquots of the buffered supernatant to give a final concentration of 50% saturation at 25°C. This amount (0.313g/ml) was determined from data presented by Green and Hughes (1955). The above mixture was stirred at 4°C for 3 hrs. and then centrifuged at 32,800g at 2°C for 15 minutes. The precipitate was discarded and the supernatant was brought to 90% saturation with ammonium sulfate and was dialyzed against 0.002M ammonium bicarbonate (Garen and Otsuji 1964) for 3 changes of 3 hrs. each. Dialysis was considered complete when no precipitate was obtained with $BaCl_2$ was added to aliquots of the dialysis solution. The dialyzate was frozen using a dry ice - acetone mixture and then lyophilized. Prior to analysis a portion of the freeze-dried material was dissolved in small amounts of 40% sucrose solution to stabilize proteins (Heitefuss *et al.* 1959). Another portion of the freeze-dried material was dissolved in deionized distilled water and used for β-glucosidase assays.

Unless otherwise stated the following designations will be used for the various extracts: 1. crude extract(s)... 'A_1', 2. dialyzed crude extract(s) ... 'A_2', 3. dialyzed crude extract(s) (from non-frozen

leaves ... 'A_3', and 4.50 - 90% $(NH_4)_2SO_4$ precipitated protein fraction(s) (partially purified protein fractions) ... 'B'.

(b) Protein Studies. Total protein determinations and electrophoretic separation of proteins were carried out on the plant extracts.

(1) Total Protein Assay: Total protein was determined quantitatively using the method described by Lowry *et al.* (1951). The standard curve was prepared using various amounts of bovine serum albumin*. Absorbance of the solutions in the early phases of the studies were determined at 750 nm using a Hitachi Perkin-Elmer spectrophotometer Model 139. In later experiments, a Beckman Model DBG spectrophotometer was used.

(2) Disc Gel Electrophoresis of Proteins: Disc gel electrophoresis was carried out applying the anionic system essentially as described by Davis (1964), using 7.0% acrylamide. Each determination was carried out in duplicate using aliquots containing 300γ of protein, as determined by Lowry *et al.* method. A current of 2 mAmp per tube was used at the beginning of the run, then increased to 3.5 mAmp per tube for the remainder of the experiment (120-150 minutes). The run was considered complete when the bromophenol blue marker band had migrated to approximately 5mm from the lower end of the tube. Protein bands were stained with 1% Naphthol blue black** in 7% acetic acid for 1 hr. The excess dye was removed by washing in 7% acetic acid. All gels were

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* Nutritional Biochemical Corporation, Cleveland, Ohio, 44128.

**Buffalo Black NBR, Allied Chemical, Industrial Chemicals Division, Morristown, N.J.

scanned using a Chromoscan* MK 11, and the pattern on each gel was recorded photographically and diagrammatically. In all diagrams of the protein bands and the isoenzyme patterns, prominent bands are shown as solid lines and faint bands as broken lines; heavily stained zones are shown as a cross-hatch, less heavily stained zones as diagonal, and very faint zones as a series of dots. The gels were stored in 7% acetic acid solution at 4°C.

(c) Enzyme Studies. The activity experiments were carried out for peroxidase, polyphenol oxidase, β-glucosidase, and catalase. As well, electrophoretic separation of isoenzymes were carried out for peroxidase, polyphenol oxidase, and esterase(s).

(1) Peroxidase: The peroxidase activity and electrophoretic separation of the isoenzymes were carried out.

(a) Activity. Peroxidase activity was measured using the procedures described in Worthington Bulletin (1967). In this procedure the rate of decomposition of H_2O_2 by peroxidase, with O-dianisidine as the hydrogen donor, was determined by measuring the rate of color development at 460 nm. Increase in absorbance was manually recorded every 15 sec. in the first set of experiments using a Hitachi Perkin-Elmer spectrophotometer Model 139, but subsequently, a Beckman DBG Model spectrophotometer with a Beckman 10" recorder was employed. One unit of peroxidase specific activity was considered to be that decomposing one micromole of peroxidase per minute per mg of protein.

(b) Electrophoretic Separation of Peroxidase Isoenzymes. The peroxidase isoenzymes were separated electrophoretically using Davis (1964) method as described previously. Then they were detected on the acryla-

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*Joyce, Loebl & Co. Ltd., Pricesway, Team Valley, Gateshead 11, England.

mide gels using the procedures described in enzyme analysis 'Special Subject', Canal Industrial Corporation, 4935 Cordell Ave., Bethesda 14, MD. Peroxidase isoenzymes were detected as colored bands after the gels were placed in a solution containing H_2O_2 and benzidine dihydrocholide reagent. The gels were incubated for 30-40 minutes in the above reagent and then washed with water and stored in 7% acetic acid at 4°C.

(2) Polyphenol oxidase(s): The polyphenol oxidase(s) activity and electrophoretic separation of the isoenzymes were determined.

(a) Activity. Initially, PPO activity assays were carried out according to the method described in Worthington Bulletin (1967) using tyrosine as substrate; however, with this procedure PPO activity could not be detected. Subsequently the method of Hall *et al.* (1969) was used. In this procedure, which involves the use of ferrocyanide and catechol, ferricyanide is produced, and the increase in absorbance at 420nm, the result of ferricyanide formation is proportional to PPO activity. A unit of polyphenol oxidase specific activity is that oxidizing one micromole substrate per minute per mg of protein.

(b) Electrophoretic Separation of Polyphenol oxidase Isoenzymes. Polyphenol oxidase isoenzymes were separated electrophoretically as described before. The isoenzymes were detected on the gels using Jolley and Mason (1965) procedure, which employs catechol-proline reagent, after a slight modification. Best results were obtained by incubating the gels in 0.1M phosphate buffer pH 6.5 for one hour and then transferred to the catechol-proline reagent for 2-3 days in the dark at room temperature (21 ± 1°C). Gels were stored in the reagent solution at 4°C. The reaction can be summarized

(Mason and Peterson 1955) as follows:

$$\text{catechol} \xrightarrow[[O]]{\text{tyrosinase}} \text{O-quinone}$$

O-quinone + L-proline + catechol → colored coupled compound.

(3) β-Glucosidase Activity: The procedures used for detecting β-glucosidase activity is essentially as described in Worthington Bulletin (1967). In this method the substrate, Salicin, is hydrolyzed by β-glucosidase to yield salignin and β-D glucose. The rate of glucose formation was determined using the method of Ashwell (1957), which is a modified method described by Nelson (1944). In this method the intensity of the color was read at 520nm and the absorbance of the reaction mixture was proportional to glucose concentration.

A unit of β-glucosidase specific activity is that liberating one micromole of glucose per minute per mg of protein.

(4) Electrophoretic Separation of Esterase Isoenzymes: Esterase isoenzymes were separated electrophoretically on acrylamide gels. Esterase isoenzymes were then detected using the procedures described by Markert and Hunter (1959) and presented in Enzyme analysis. 'Special Subject', Canal Industrial Corporation. In this procedure gels were incubated in a reagent containing alphanaphthylacetate and fast blue RR* and isoenzymes bands were detected after 30-40 minutes. The gels were washed with deionized distilled water and stored in 7% acetic acid at 4°C.

(5) Catalase Activity: Catalase activity was measured spectrophotometrically using the method described by Beers and Sizer (1952) and

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* Diasotate - 4 - benozylamino - 2,5 - dimethoxy aniline. Sigma Chemical Company, St. Louis, Missouri.

described also in Worthington Bulletin (1967). The decrease of absorbance of H_2O_2 at 240nm was recorded and the slope of the initial straight line was determined. One unit of enzyme activity was considered to be equal to one micromole of H_2O_2 decomposed per minute per mg of protein.

RESULTS

1. Reaction of Redwing and S-66-53 to *Melampsora Lini* Race '300'.

Variety Redwing gives a susceptible reaction to race '300'; whereas, S-66-53 is immune and therefore not even a conspicuous flecking would be observed up to 9 days after inoculation, (Fig. 1).

2. Cytological Studies

The interaction between each of Redwing and S-66-53, and rust fungus *Melampsora lini* was studied by light and electron microscopy, (Figs. 2-15).

Key to abbreviations used in Figures 2-15

c: chloroplast

cf: fungus cytoplasm

ch: host cytoplasm

chc: collapsed host cell

cu: cushion-like structure

d: dark stained bodies

e: encapsulation zone

ef: fungus endoplasmic reticulum

eh: host endoplasmic reticulum

fc: fungus cell

gt: uredospore germ tube

h: haustorium

hc: host cell

hf: hyphal fragment

Fig. 1. Reaction of Redwing and S-66-53 to inoculations with *M. lini* 'race 300' uredospores. 'A' and 'B' indicate uredospore sori, and remains of uredospores used in inoculations respectively.


A
B
Redwing
S-66-53

hm: haustorial mother cell

hn: haustorial neck

i: intercellular mycelium

l: lamellar structure

mf: fungus mitochondrion

mh: host mitochondrion

mm: multiple membrane structure

nf: fungus nucleus

nh: host nucleus

nm: nuclear membrane

nu: nucleolus

pa: paraphysis

pf: fungus plasma membrane

ph: host plasma membrane

pt: penetration tube

o: oil globules

sg: starch grain

st: stoma

u: uredospore

ud: uredium

uw: uredospore wall

v: vesicle

vf: fungus vacuole

vh: host vacuole

wf: fungus cell wall

wh: host cell wall

(a) Light Microscopy. The results for light microscopy studies of non-inoculated cotyledons of 9-day-old Redwing (susceptible) and S-66-53 (immune) seedlings as well as for 2-, and 9-day-old inoculated cotyledons (i.e. cotyledons of 11- and 18-day-old seedlings), are shown in Figs. 2-9.

Germ tubes of germinated uredospores were found on the epidermis of Redwing cotyledons two days after inoculations (Fig. 2C and D), and host cells appeared normal, comparable to cells studied in sections of non-inoculated cotyledons (Fig. 2A and B). Intercellular fungal growth and the haustoria were quite prominent nine days after inoculations (Fig. 3). Host cells at this stage were to a large extent devoid of nuclei and chloroplasts. In some cells however, chloroplasts appeared to be clustered together (Fig. 3B). Also few host cells appeared to be collapsed (Fig. 3F). Uredia containing uredospores with thick spore wall, dense cytoplasm, and oil globules and one or two nuclei were found in some sections of 9-day inoculated cotyledons (Fig. 4).

Uredospores germinated on the epidermis and penetration tubes entered the leaf through the stomata in S-66-53 and Redwing (Fig. 5D and 7B). Very limited intercellular fungal growth was observed in studied sections of S-66-53 cotyledons two days after inoculations (Fig.6). This observed intercellular hyphae, which constituted of larger but fewer cells than those observed in Redwing, contained what appeared to be oil

Fig. 2. Redwing, non-inoculated and 2 days after inoculation.

(A) - Cells of the non-inoculated. X 290.

(B) - Cells of the non-inoculated. X 880.

(C) - Uredospore germination on Redwing epidermis near a stoma. Host cells appeared normal. X 180.

(D) - Enlarged portion of ('C'), close to the stoma. Note the germ tube near the stoma. Host cells contained chloroplasts with starch grains. X 1600.

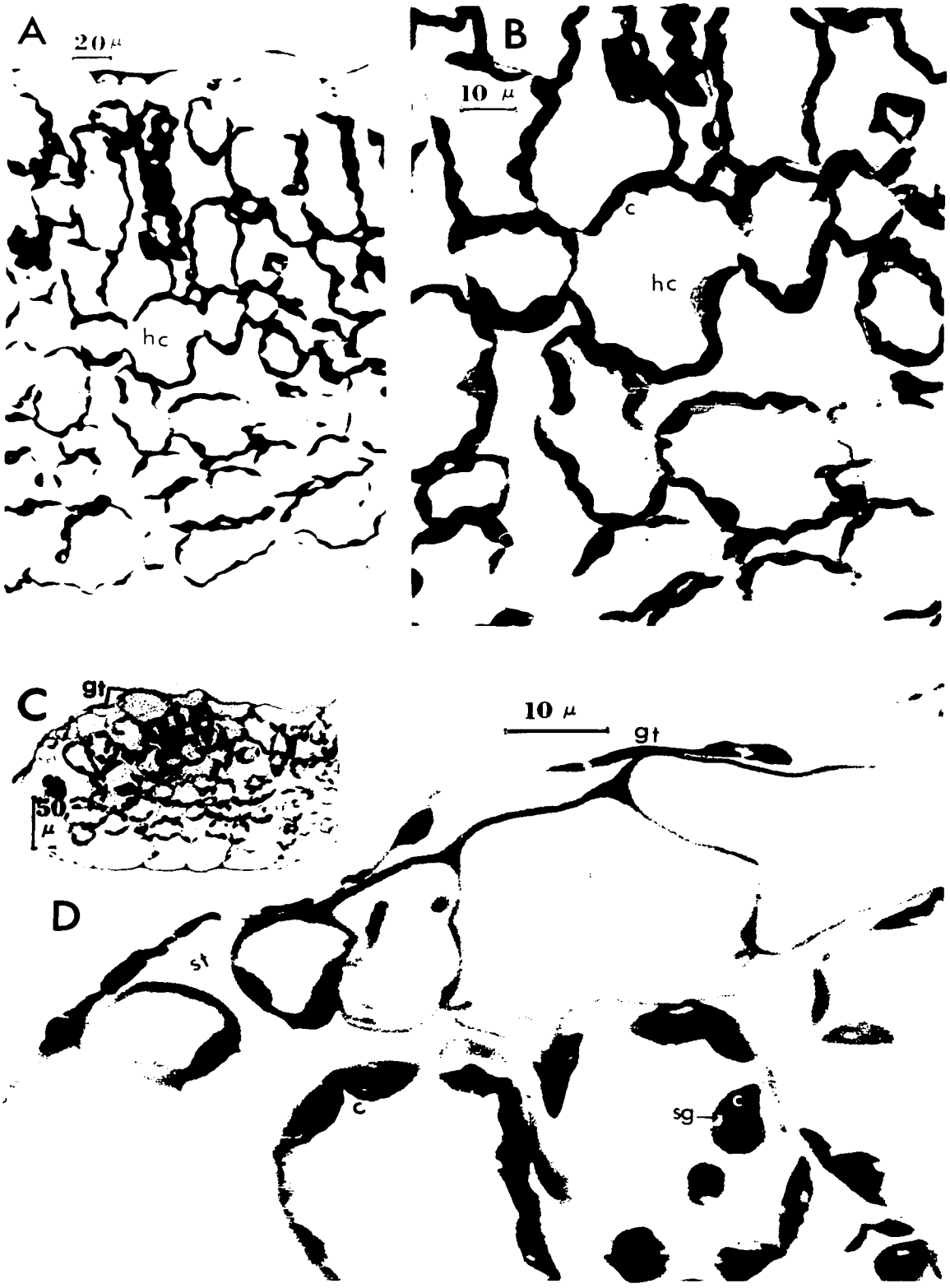
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Fig. 3. Redwing, 9 days after inoculation.

(A) - Cross section of sections in inoculated cotyledon. Note prominent hasutoria and intercellular mycelium. X 160.

(B) - A host cell with clustered chloroplasts and haustoria. Note intercellular mycelium. X 1490.

(C) - Host cells devoid from all organelles but contained haustoria. Intercellular mycelium is evident. X 1100.

(D) - Host cells with haustoria of which one appeared to be connected with its haustorial neck is seen. X 1480.

(E) - Host cells, near a stoma, with few chloroplasts and haustoria. One haustorium appeared attached to a haustorial neck. X 1140.

(F) - Collapsed host cells and extensive intercellular mycelium. X 1200.

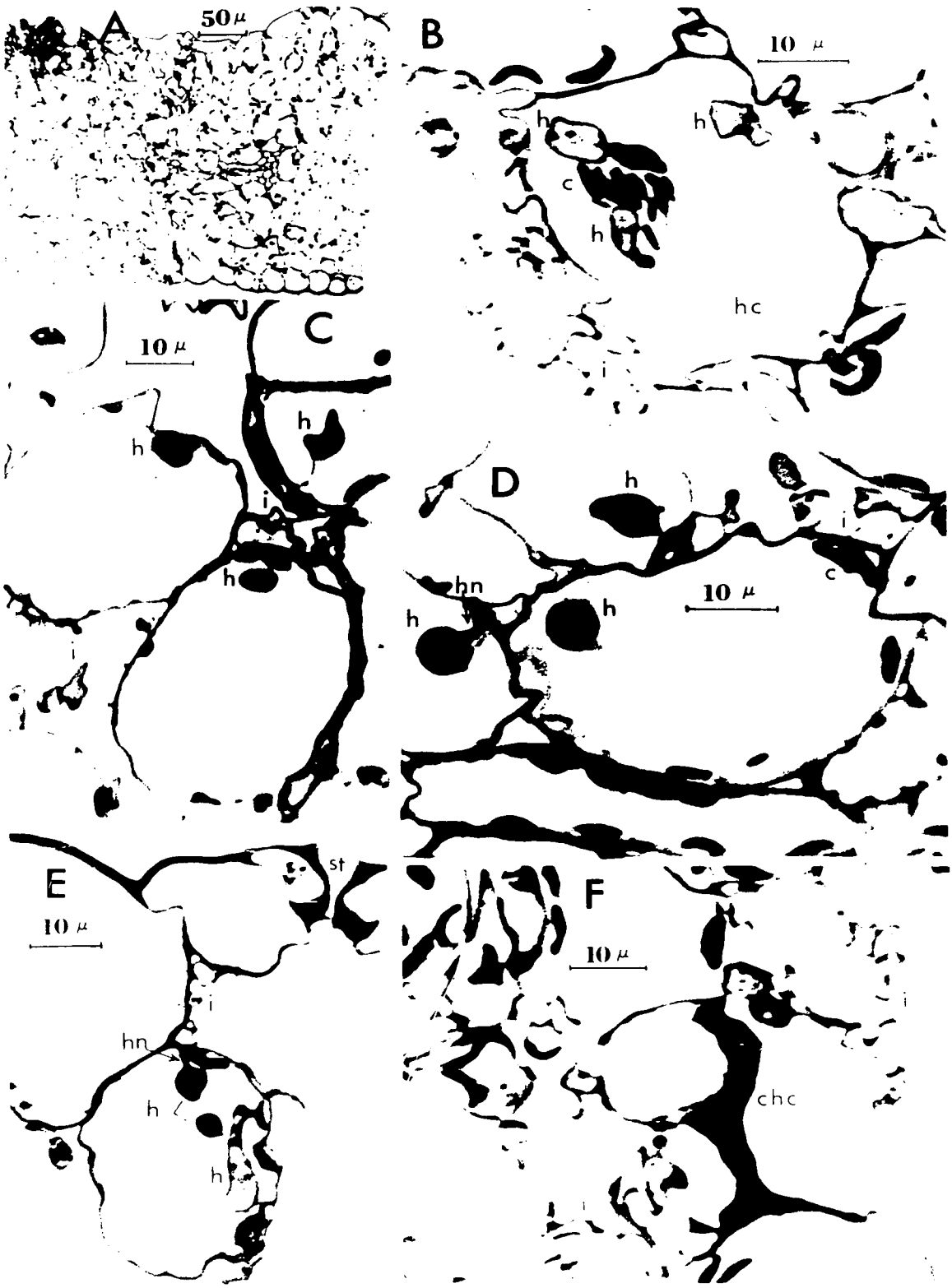
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Fig. 4. Redwing, 9 days after inoculation. The development of uredia and uredospores.

(A) - Cross section of a uredium showing uredospores, paraphysis, and dark stained collapsed host cells. X 240.

(B) - Uredospores with thick wall, dense cytoplasm, and oil globules. One or two nuclei are seen in some uredospores. X 1530.

(C) - Enlarged portion of (A) showing uredospores intercellular mycelium and collapsed host cells. X 640.

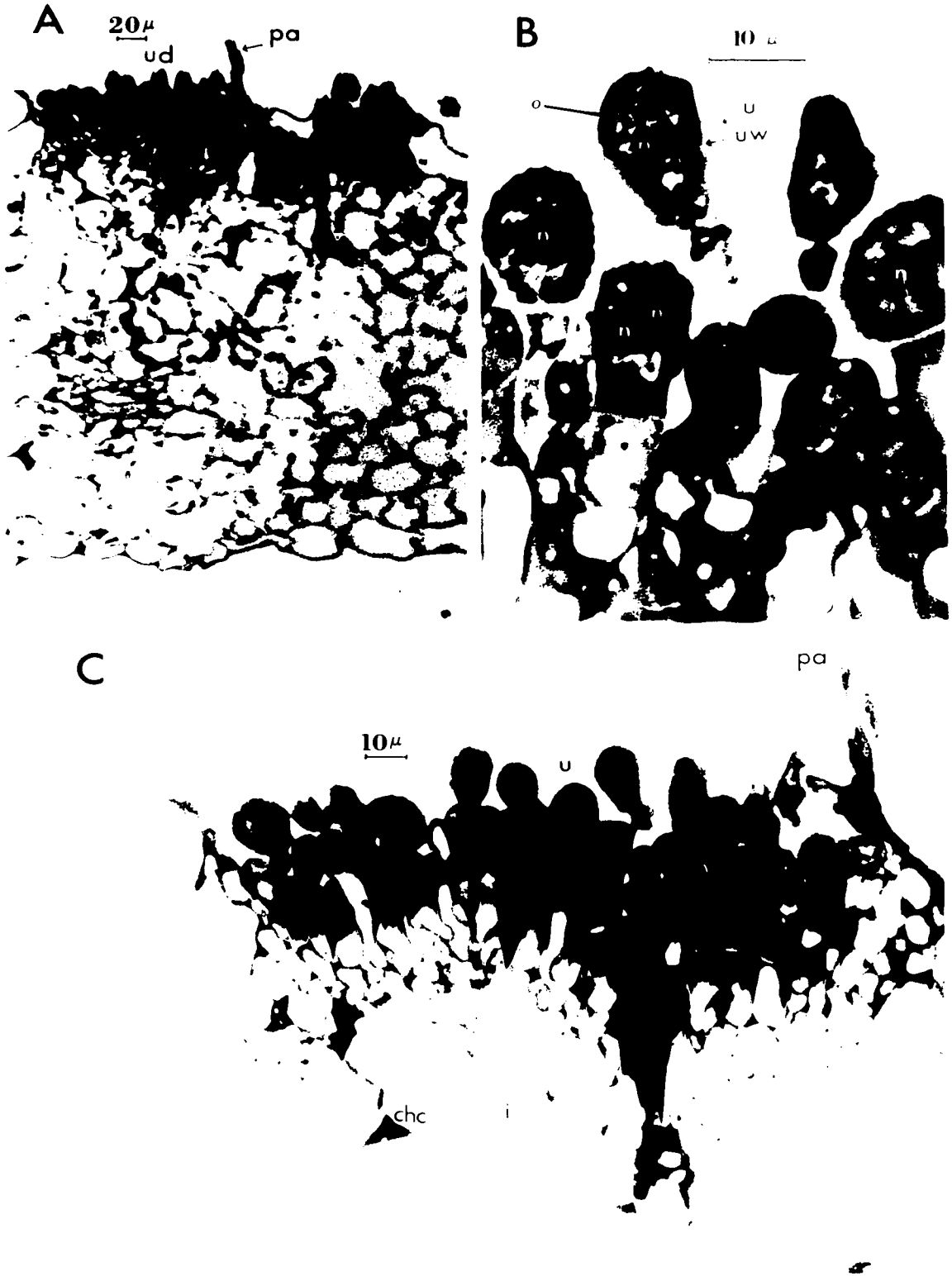
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Fig. 5. S-66-53, non-inoculated and 2 days after inoculation.

(A) - Cells of the non-inoculated. X 200.

(B) - Cells of the non-inoculated. X 850.

(C) - Cross sections in 2-day inoculated cotyledons showing two collapsed host cells (1 and 2) near the epidermis. 1 and 2 are enlarged in (D) and (E) respectively. X 360.

(D) - Cross section in 2-day inoculated cotyledons showing a germinated uredospore, penetration tube entering through a stoma and a dark stained collapsed host cell, (1). X 830.

(E) - A dark stained collapsed host cell (2). Adjacent to this cell (2) another host cell showed swollen chloroplasts with starch grains. Note that the rest of the host cells appeared normal. X 1280.

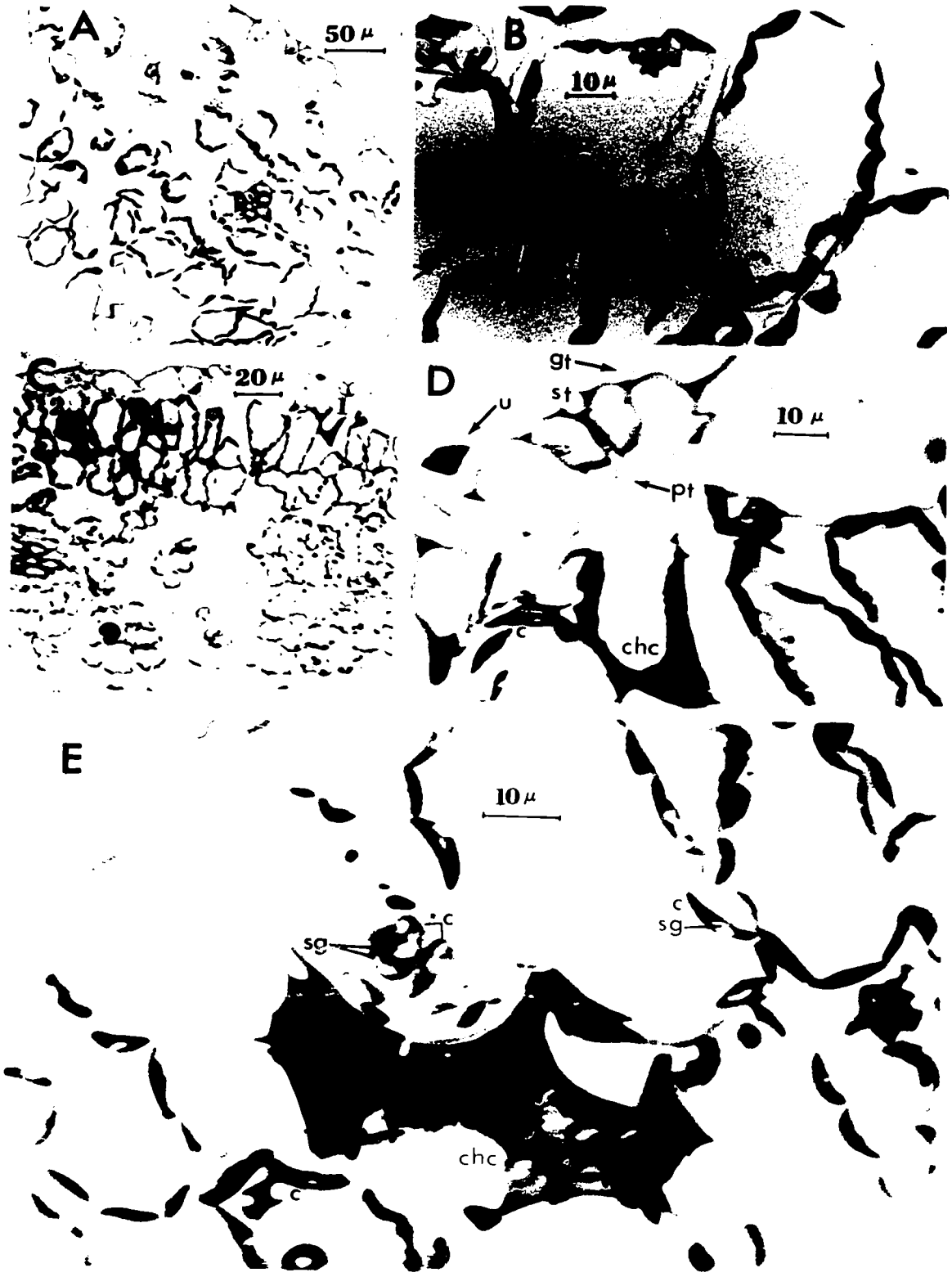
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Fig. 6. S-66-53, 2 days after inoculation.

(A) - A cross section showing two hyphal fragments and a collapsed host cell near a stoma. X 160. This section was stained only for 15" with toludine blue instead of one minute.

(B) - Enlarged portion of (A) showing the larger hyphal fragment and the dark stained collapsed host cell. The hyphal fragment contained vacules, dense cytoplasm and oil globules. Note the collapsed host cell with a dark cytoplasm and chloroplasts. X 2180. Adjacent host cells appeared normal.

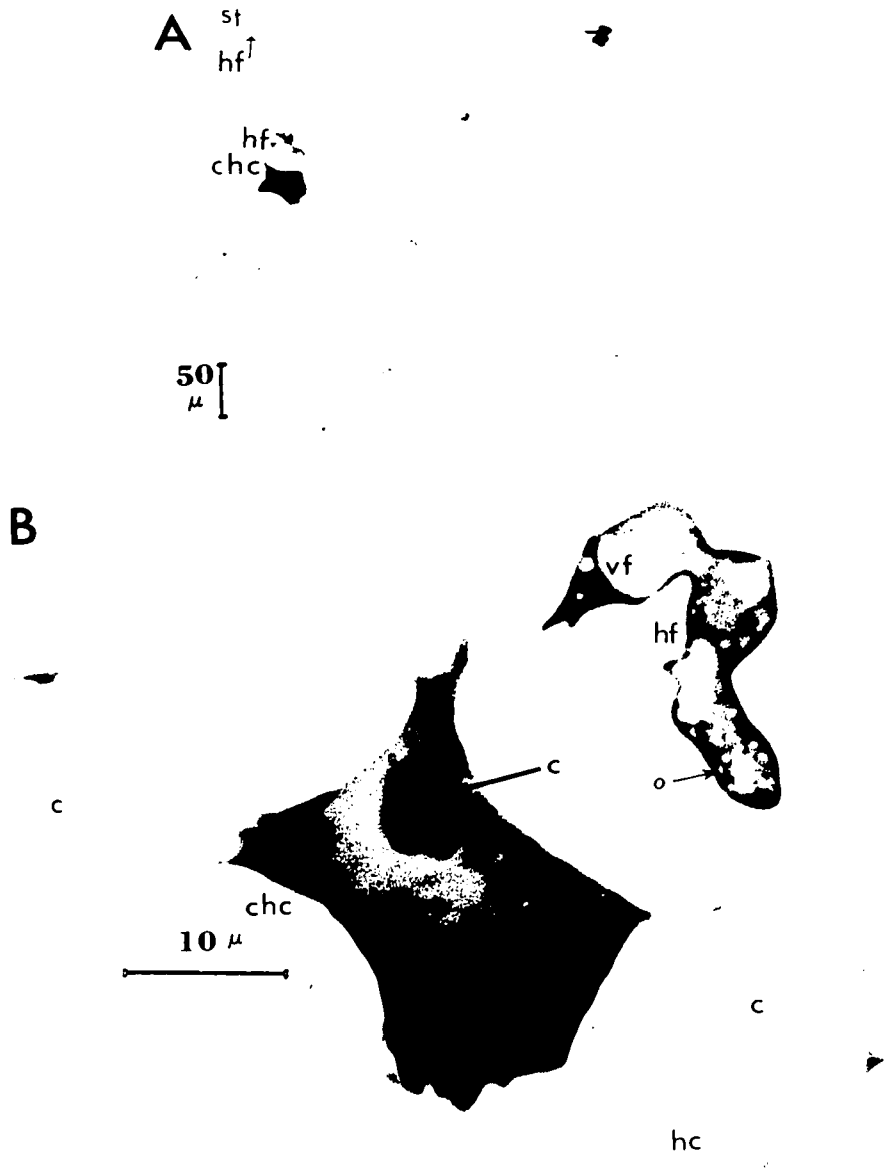
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Fig. 7. S-66-53, 9 days after inoculation.

(A) - Cross section showing a germinated uredospore entering through a stoma, and two host cells (1 and 2) slightly degenerated. '1' and '2' are shown in (C). X 210.

(B) - Enlarged portions of A showing a germinated uredospore with the penetration tube entering through a stoma. X 1520.

(C) - Enlarged portion of A showing two cells '1' and '2' slightly degenerated. These two cells contained swollen chloroplasts with large starch grains. Note the necrotic cell walls in the zone of contact between these two cells. X 1690.

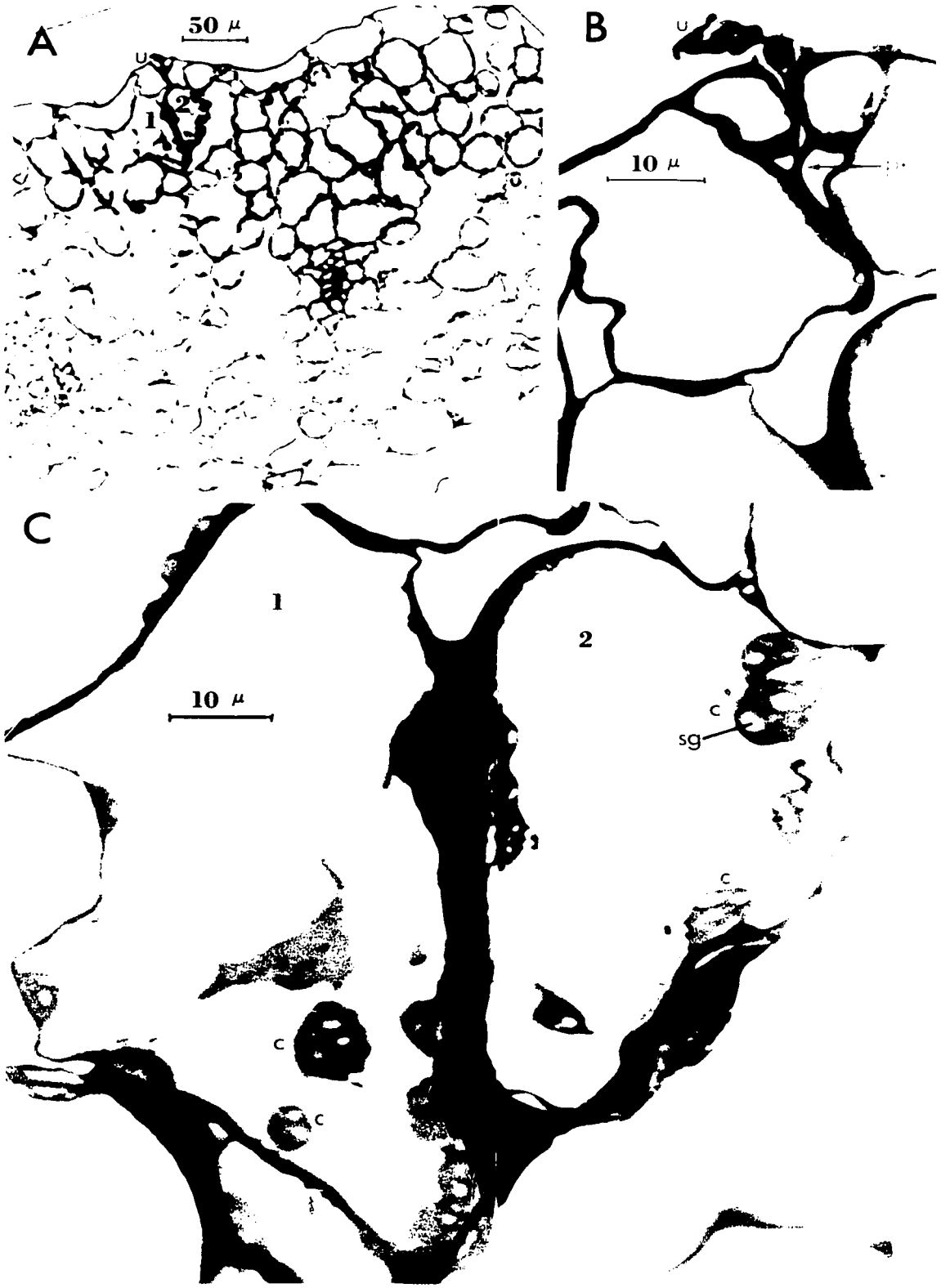
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Fig. 8. S-66-53, 9 days after inoculation.

(A) X 950, (B) X 970, (C) X 980, (D) X 970, (E) X 950, (F) X 950. Serial reactions in the proximity of the site of infection shown before in Fig. 6. Note the cells marked 1 and 2 in Fig. 6 are also marked in (B). Cells contained swollen chloroplasts with large starch grains. Serial sections in a collapsed host cell are seen.

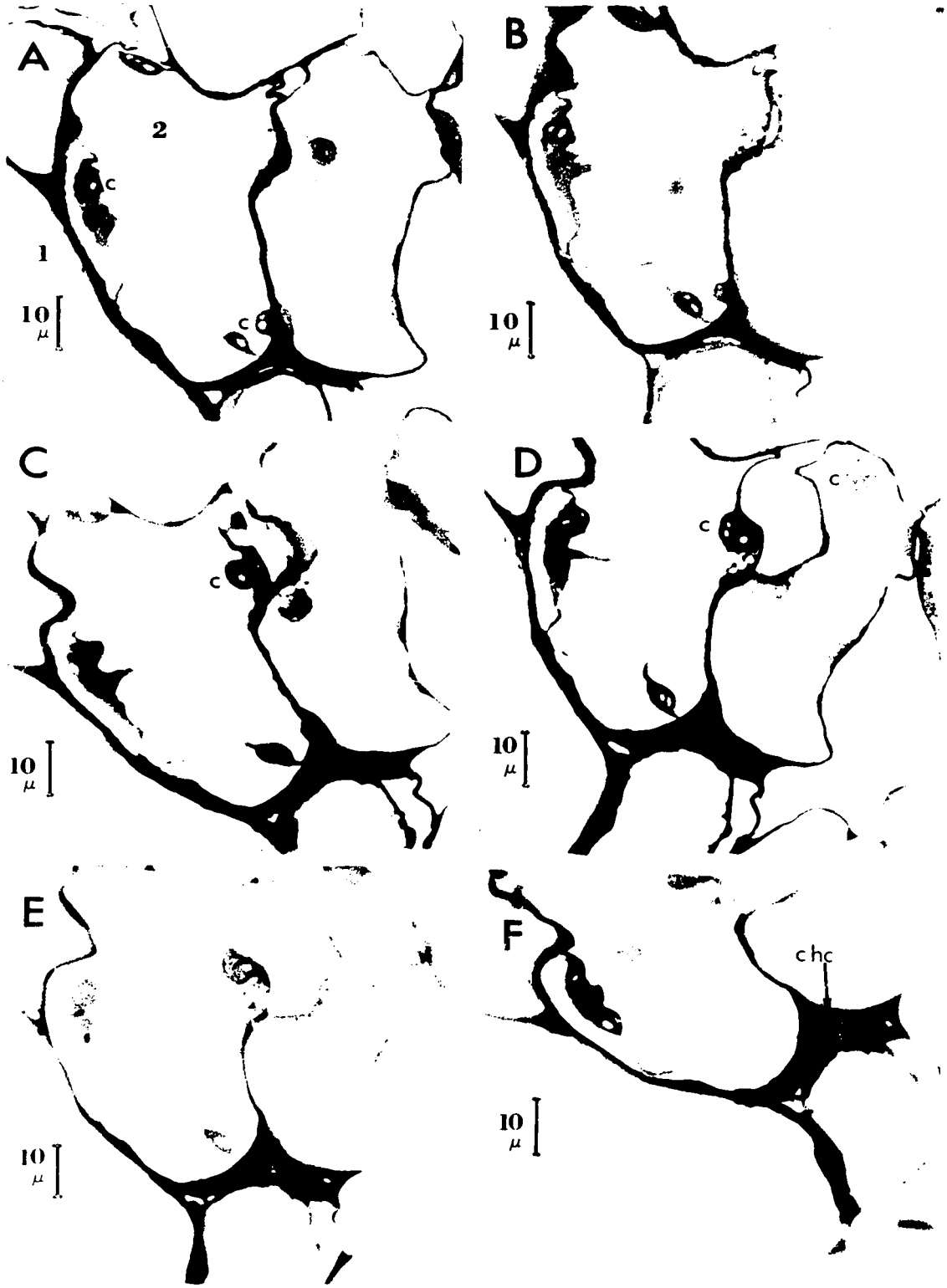
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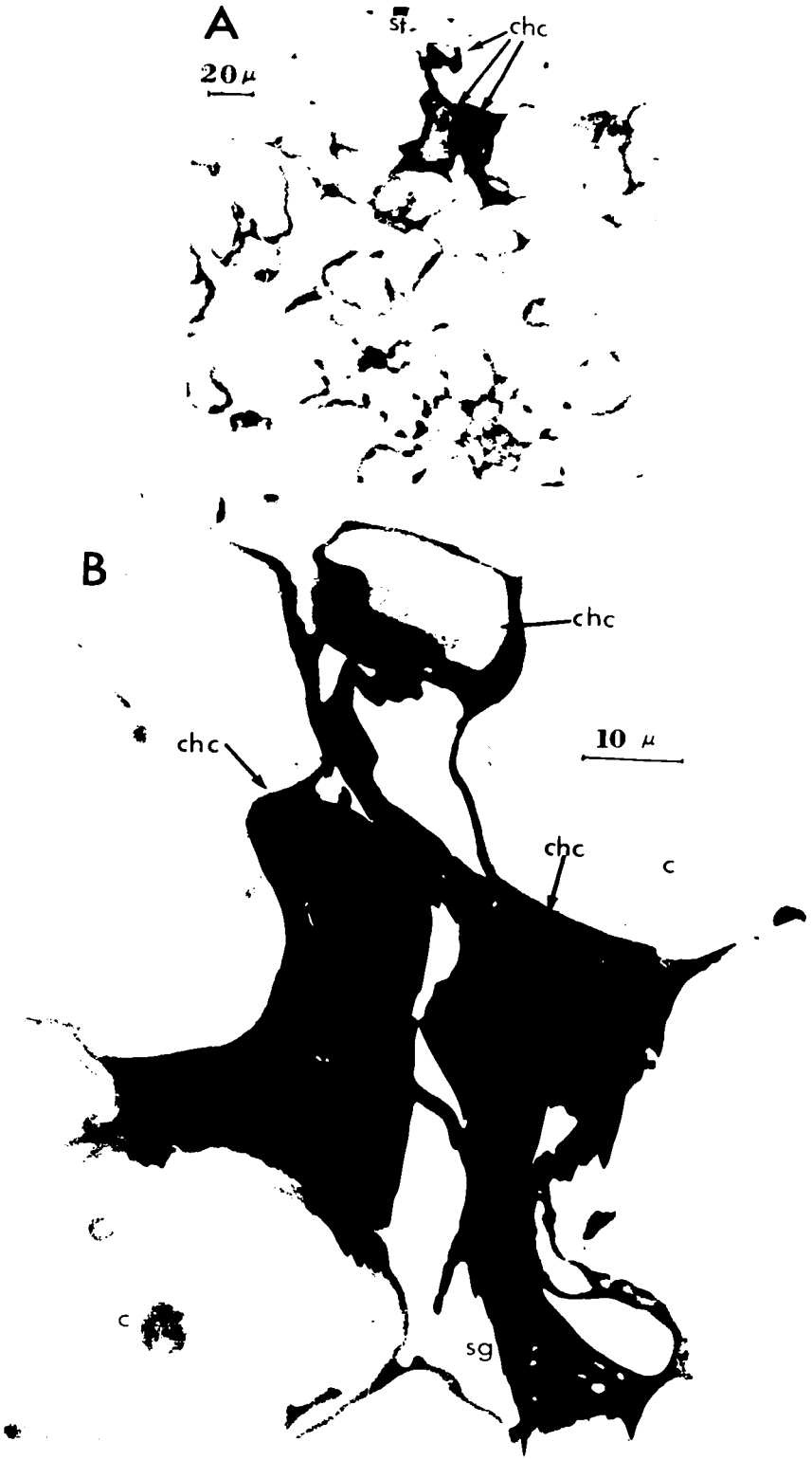
Fig. 9. S-66-53, 9 days after inoculation.

(A) - Cross section in an inoculated cotyledon showing three collapsed host cells near a stoma. X 320.

(B) - Enlarged portion of A showing the three collapsed host cells. One of the cells appears very dark stained and contains starch grains. Few adjacent host cells contained swollen chloroplasts. X 1480.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globules and it was thicker than hyphae observed in Redwing. Few host cells at this stage, usually near stomata, showed signs of degeneration and they took toludine blue stain more readily than the rest of the cells (Fig. 5). However, when sections were stained only for 15 seconds instead of one minute, chloroplasts were revealed in these affected host cells (Fig. 6). Adjacent host cells did not show signs of degeneration. In S-66-53, 9 days after inoculation, very limited necrotic areas in the proximity of stomata were observed (Figs. 7-9). Chloroplasts seemed to be swollen in host cells near the site of infections (Figs. 7-9) in which fungus mycelium was not observed; however necrotic cell walls were observed in some affected cells (Fig. 7). Several sections of the same site of infections revealed a collapsed host cell (Fig. 8). Fig. 9 shows also few collapsed host cells near a stomata through which the fungus has entered.

(b) Electron Microscopy. Electron micrographs showed, in more detail than light microscopy, the interaction between each of the two hosts and the parasite. These results are shown in Figs. 10-15.

A nucelus with a nucleolus of a non-inoculated cotyledon of Redwing is shown in Fig. 10A. This nucleus was delineated by a double membrane. Also, shown in Fig. 10A are, chloroplasts, mitochondrions, and endoplasmic reticulum. The normal lamellar structure of a chloroplast (at a higher magnification than in Fig. 10A) of the non-inoculated cotyledons is shown in Fig. 10B.

A uredospore germ tube appressed to epidermis, var. Redwing, two days after inoculation is shown in Fig. 10C and D. The germ tube, as it elongated, appeared to fold upon itself. This germ tube had a thick wall and it contained dense cytoplasm with numerous oil globules. Nine days

after inoculation, the intercellular mycelium was extensive. Haustorial mother cells were observed in close contact with the host cell wall (Fig. 11A, B, C, and D). The inner cell wall of the haustorial mother cell and the cell wall of the host cells at the zone of contact were much thicker than walls removed from the penetration site (Fig. 11B and D). Mitochondria (Fig. 11B and D), and a multiple membrane structure (Fig. 11B) were observed in the haustorial mother cell. Profiles of haustorial necks were observed in some sections (Fig. 11A, B and D). The haustorial neck was surrounded with a wall continuous with that of the haustorial mother cell. A sheath, continuous with the host cell wall, was observed around a haustorium neck (Fig. 11B). The extent of the sheath, observed, in Fig. 11B, was not observed in Fig. 11D. The haustorial necks appeared to be encapsulated (Fig. 11B, and D). Host plasma membrane can be seen continuous around the sheath (Fig. 11B). Cytoplasmic material appeared to be passing from the mother cell into the developing haustorium with the result that the mother cell was nearly empty (Fig. 11B and D). Electron micrographs of a cross section of an encapsulated haustorium proper is shown in Fig. 11C, and E. In the outer layer of the encapsulation of this haustorium, vesicle structures were observed (Fig. 11E). Also, endoplasmic reticulum was evident around this encapsulated haustorium in which mitochondria and dark stained bodies were observed; and haustoria appeared to have numerous ribosomes. In Fig. 12A and B fungal and host cell, adjacent to one another, appeared to have cell walls, of uniform thickness i.e. there was not a thickened portion at the zone of contact. However, a cushion-like structure was observed between these cells (Fig. 12A and B). The dikaryotic nature of the fungus at this part of the life cycle was revealed in some haustoria containing two

nuclei (Fig. 12C and D). At this stage of infection host cells containing chloroplasts with abnormal lamellar arrangements (Fig. 11A and B, and 12A, B, C, and D) compared to that of the non-inoculated cotyledons (Fig. 10A and B). A multiple membrane structure was observed in a host cell (Fig. 12B).

The electron micrographs of sections of non-inoculated and inoculated cotyledons of S-66-53 are shown in Figs. 13-15.

A chloroplast with the normal lamellar structure, of a non-inoculated cotyledon is shown in Fig. 13A. Mitochondria and multiple membrane structures are shown also in Fig. 13A.

In a section of a 2-day inoculated cotyledon, corresponding to those studied with light microscopy (Fig. 6), limited hyphal fragments were observed near a stoma (Fig. 13B). The large hyphal fragment contained a nucleus. Mitochondria, endoplasmic reticulum, vacuoles, and multiple membrane structures can be seen (Fig. 13C, D, and E). The smaller hyphal fragment showed a large vacuole, dense cytoplasm with numerous mitochondria, oil globules, and a few multiple membrane structures (Fig. 14A and B). In S-66-53 variety a collapsed host cell close to the smaller hyphal fragment (Fig. 13B) contained cytoplasm which was stained darker than surrounding cells. This necrotic cell contained chloroplasts with starch grains and distorted lamellar structures (Fig. 14C and D)(these abnormal chloroplasts differed from chloroplasts observed in inoculated cotyledons of Redwing (Fig. 11 and 12)).

The sections of 9-day-old inoculated cotyledons showed few collapsed host cells Fig. 15, usually near stomata as in two days after inoculations, with numerous unstained structures that are probably starch grains. The starch grains were surrounded with a limited lamellar structure that could represent remainder of chloroplasts.

Fig. 10. Redwing, non-inoculated and 2-days after inoculation.

(A) - A portion of a cell from a non-inoculated cotyledon showing nucleus, nucleolus, chloroplasts, mitochondria and endoplasmic reticulum. The nucleus is delineated by a double membrane. X 12,200.

(B) - Lamellar structure of a chloroplast of a non-inoculated cotyledon. X 44,400.

(C) - Uredospore germ tube appressed to epidermis. Notice that the germ tube folds upon itself. X 8,900.

(D) - Enlarged portion of (C) showing the thick wall of the germ tube and dense cytoplasm with numerous oil globules. X 19,100.

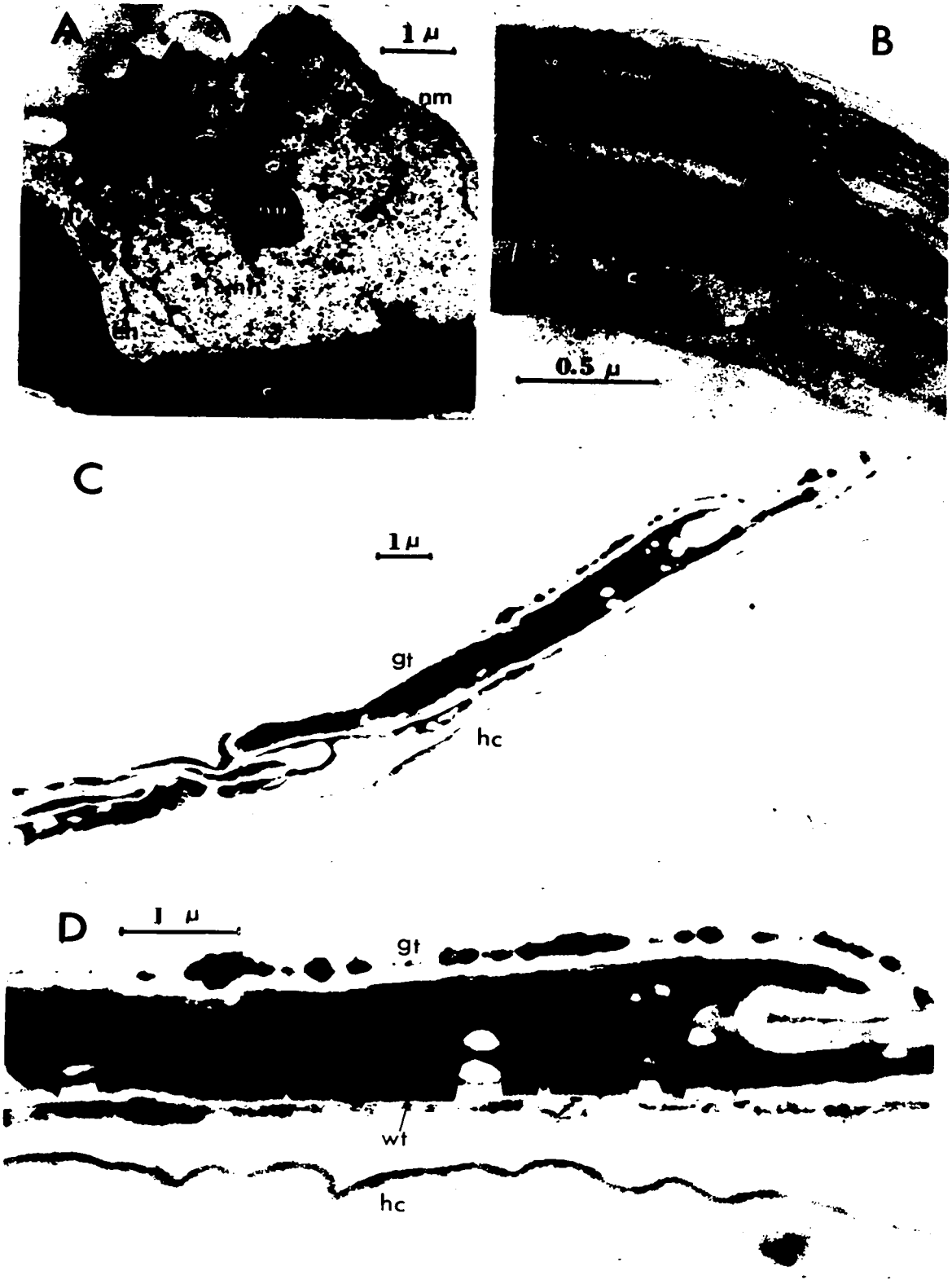
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Fig. 11. Redwing, 9 days after inoculation.

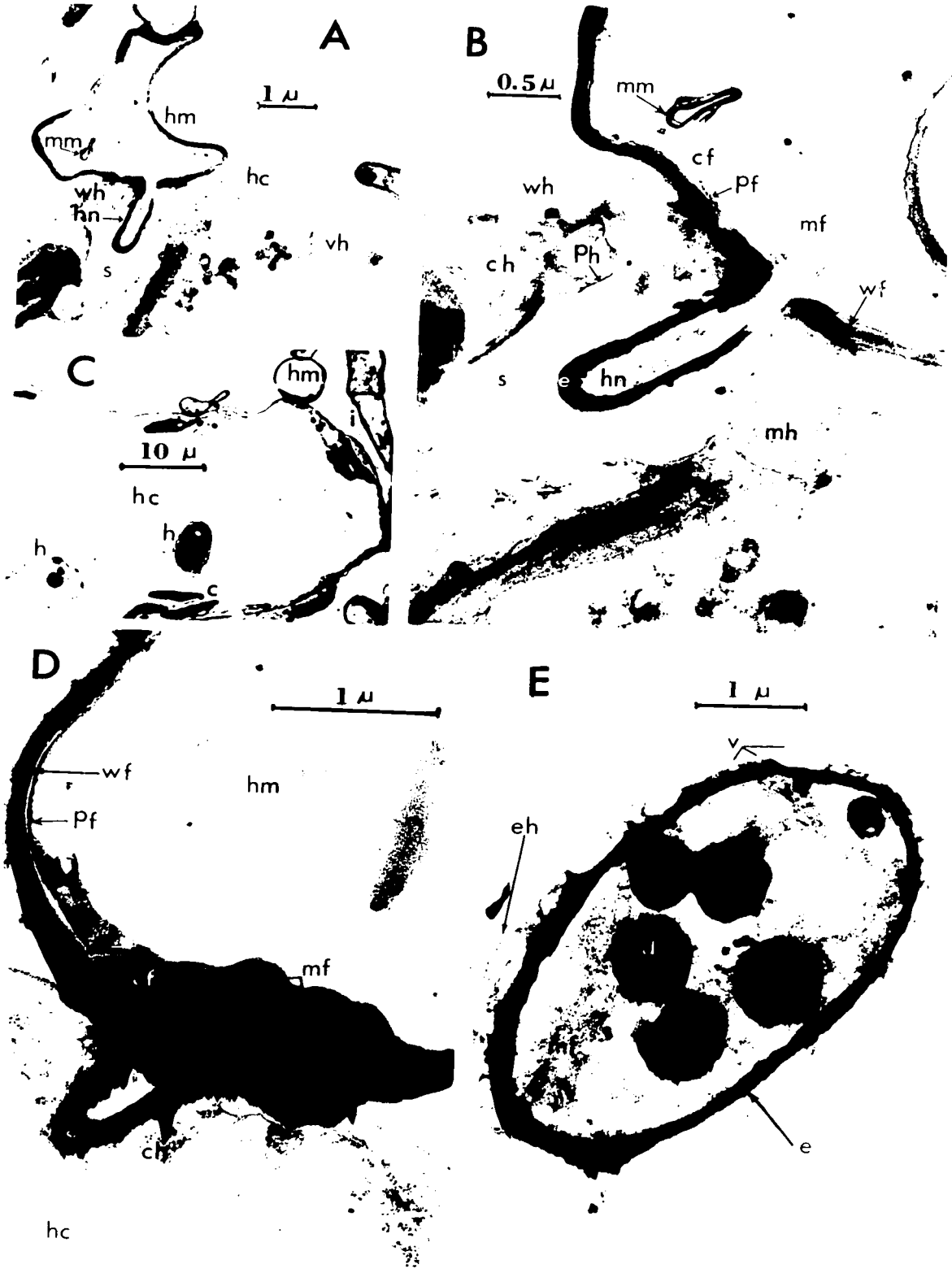
(A) - A haustorial mother cell with haustorial neck in contact with a host cell. Notice multiple membrane structure in the haustorial mother cell. X 9,500.

(B) - Enlarged portion of (A). Note thickened inner wall of the haustorial mother cell and thickened host cell wall in the zone of contact. The haustorial neck is encapsulated, and surrounded with a sheath continuous with host cell wall. Plasma membrane of the host is seen around the sheath. Cytoplasmic material appears to be passing from the haustorial mother cell into the haustorial neck. A multiple membrane structure is seen in the haustorial mother cell. Host chloroplasts have distorted lamellar structure. X 24,400.

(C) - Host cell with chloroplasts, haustoria and a haustorial mother cell resting on the upper part of the cell. X 1400.

(D) - Enlarged portion of (C) showing the haustorial mother cell with an encapsulated haustorial neck. Note thickened inner wall of the haustorial mother cell and thickened host cell wall at zone of contact. Cell material appears passing from the haustorial cell into the developing haustorium. X 27,000.

(E) - Enlarged portion of (C) showing an encapsulated haustorium with mitochondria and dark stained bodies. Note vesicles developing from the outer layer of encapsulation, and endoplasmic reticulum close to the haustorium. X 17,900.


A
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wh
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B
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C
hm
10 μ
hc
h
h
c
i
D
1 μ
wf
hm
Pf
mf
ch
hc
E
1 μ
v
eh
e

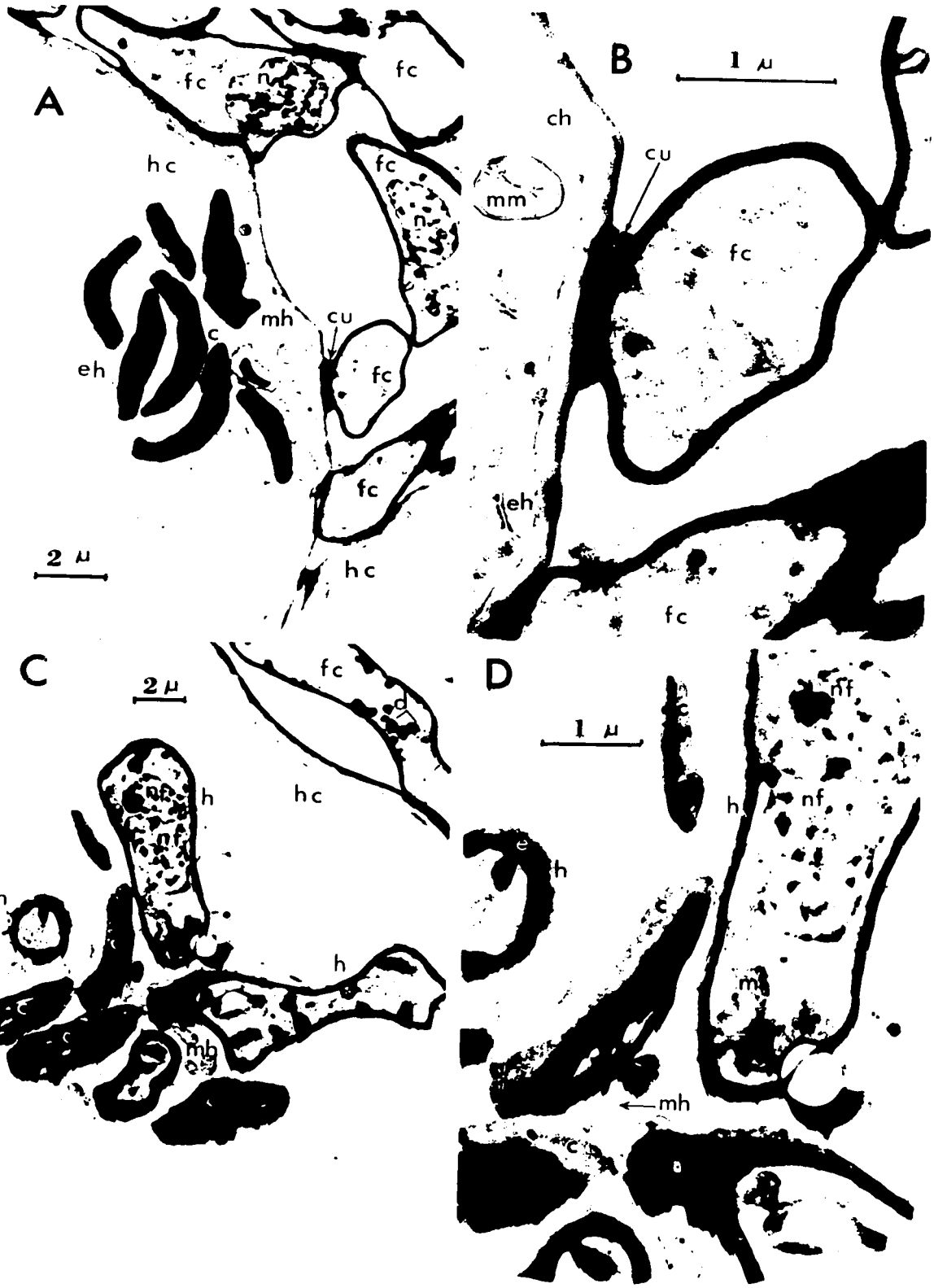
Fig. 12. Redwing, 9 days after inoculation.

(A) - Fungal cells in close proximity to a host cell. Host cell contained chloroplasts with abnormal lamellar structure. Note cushion-like structure between a fungus cell and the host cell. X 5,500.

(B) - Enlarged portion of 'A' showing a fungal and host cell, adjacent to one another. Cushion-like structure is seen between two cells that have cell walls of even thickness. Note a multiple membrane structure in the host cell. X 26, 200.

(C) - Portion of host cell contained, altered chloroplasts, mitochondria and haustoria of which one contained 2 nuclei. Fungal cell with dark stained body is seen at the upper part. X 4,200.

(D) - Enlarged portion of C showing a part of a host cell cytoplasm with haustoria containing mitochondria, chloroplasts with distorted lamellar structure and mitochondria. X 16,500.


A
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fc
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2 μ
B
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C
2 μ
fc
hc
h
h
h
mh
D
1 μ
nf
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nf
h
mh

Fig. 13. S-66-53, non-inoculated and 2 days after inoculation.

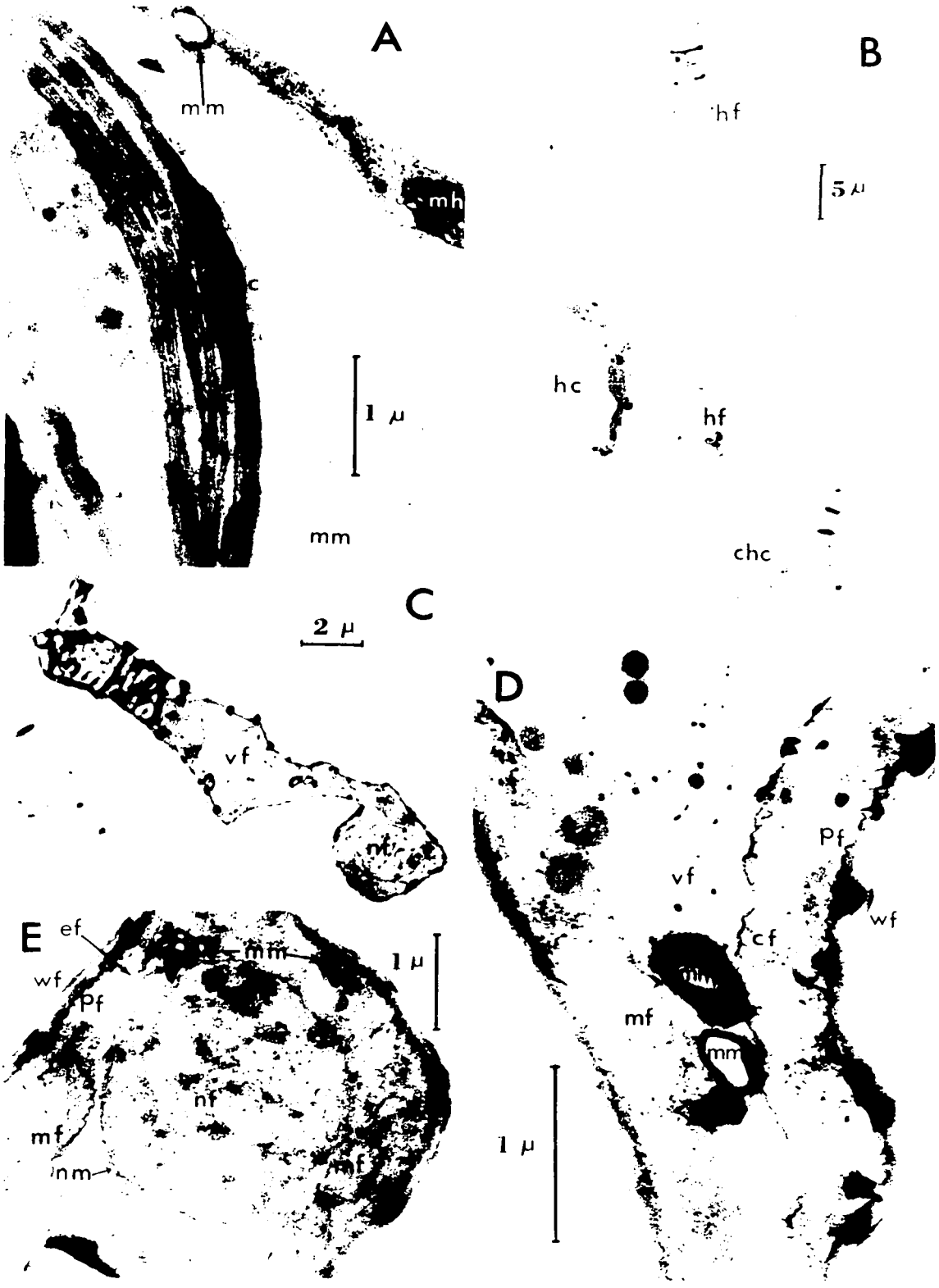
(A) - Chloroplast with lamellar structure, mitochondria and multiple membrane structure in the cytoplasm of a cell of a non-inoculated cotyledon. X 21,000.

(B) - Cross section in a 2-day inoculated cotyledon near a stoma. Note two hyphal fragments and a part of a collapsed host cell. X 2,000.

(C) - Enlarged portion of 'B' showing the larger hyphal fragment with a vacuole and a nucleus. X 5,000.

(D) - Enlarged portion of the central part of the above hyphal fragment showing mitochondria and multiple membrane structures and a large vacuole. X 31,100.

(E) - Enlarged portion of C showing a nucleus, and dense cytoplasm with mitochondria, endoplasmic reticulum, and multiple membrane structures. X 17,000.


A
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mh
c
1 μ
mm
B
hf
5 μ
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chc
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D
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mf
mm
1 μ
E
ef
mm
1 μ
wf
Pf
nf
mf
nm
mf

Fig. 14. S-66-53, 2 days after inoculation.

(A) - Enlarged portion of Fig. 12 'B' showing the smaller hyphal fragment containing a large vacuole, dense cytoplasm with mitochondria and oil globules. Note multiple membrane structures close to the cell wall. X 11,100.

(B) - Enlarged portion of 'A' showing the multiple membrane structures. X 65,300.

(C) - Enlarged portion of Fig. 12 'B' showing a collapsed host cell with a dark stained cytoplasm and chloroplasts with starch grains. X 4,100.

(D) - Enlarged portion of the above collapsed host cell showing a chloroplast with a starch grain and distorted lamellar structure. X 29,100.

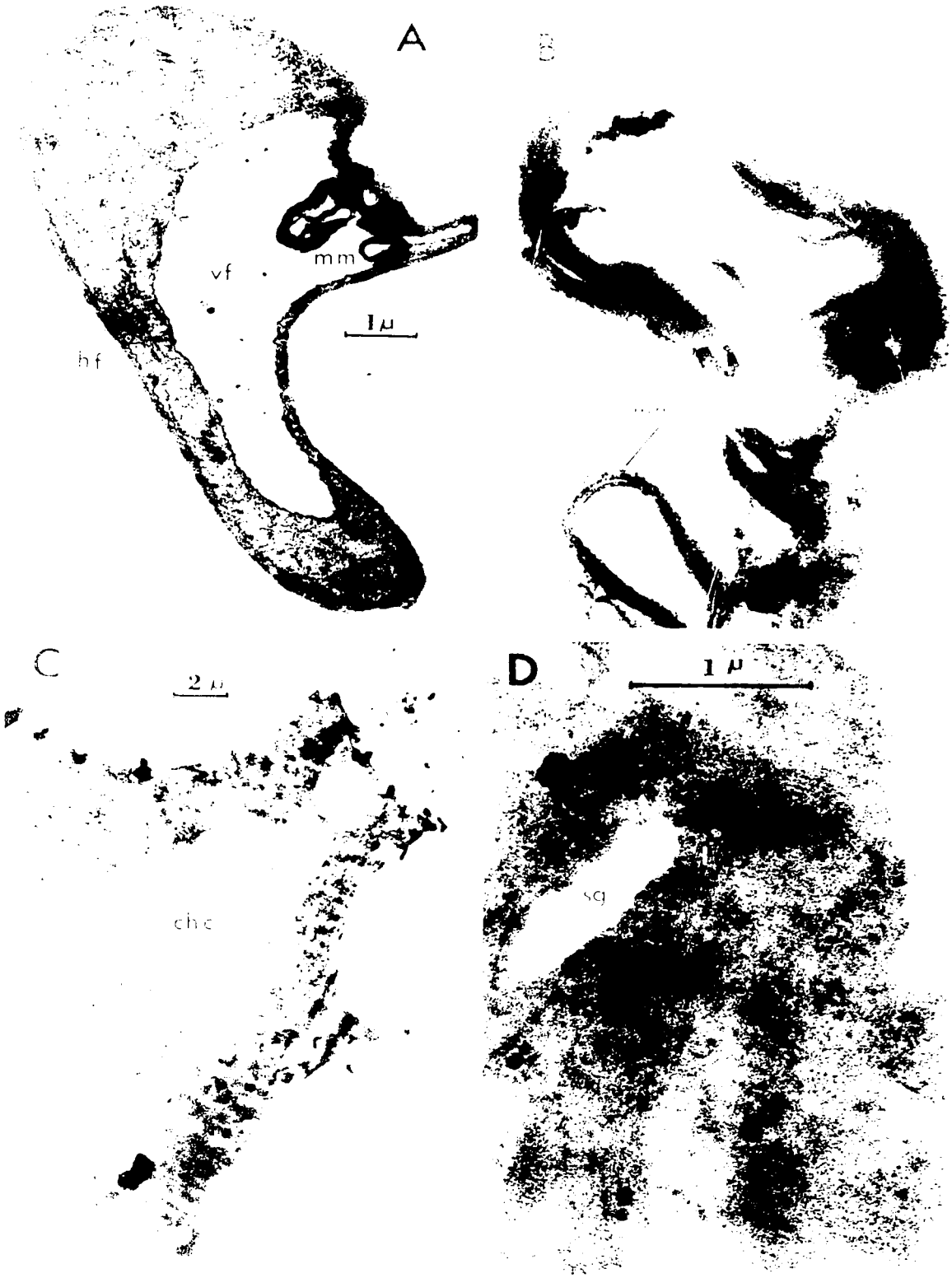
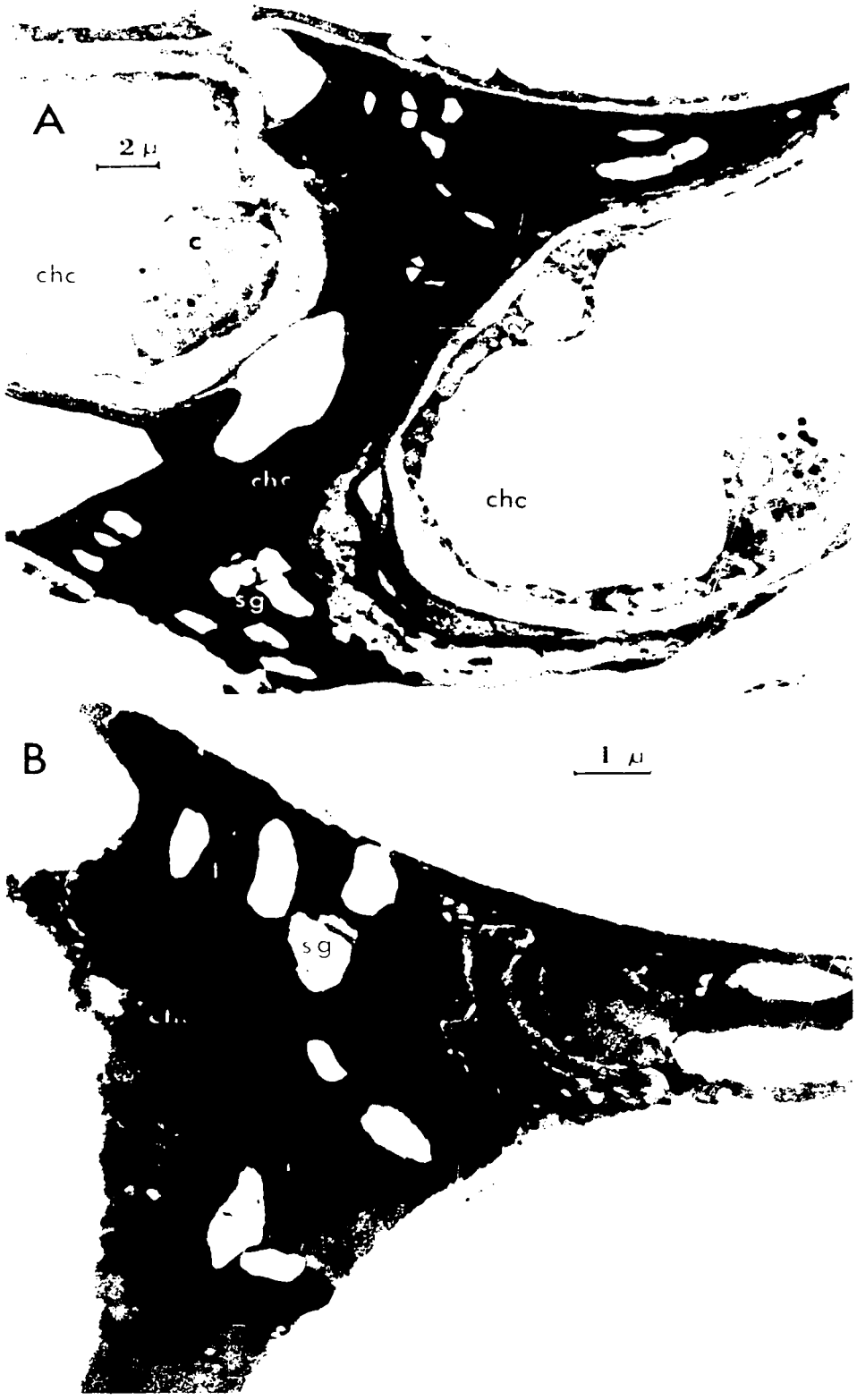

A
B
mm
vf
1μ
hf
C
2μ
chc
D
1μ
sg

Fig. 15. S-66-53, 9 days after inoculation.

(A) - A collapsed host cell appears very dark stained. Note the unstained starch grains. X 4,500.

(B) - Enlarged portion of 'A' showing part of the above collapsed cell. Starch grains appear to be surrounded with a limited lamellar structure that might represent remains of chloroplasts. X 11,400.


A
2 μ
c
chc
chc
chc
sg
B
1 μ
sg

In all of the studied sections of 2-, 9-day inoculated cotyledons of S-66-53, structures that represented haustoria were not observed.

3. Immunological Studies.

Precipitin lines were not obtained when sera removed from rabbits, prior to immunization, were reacted with antigens extracted from either Redwing or S-66-53. Therefore, all precipitin lines observed in the following tests are due to a specific antigen - antibody reaction.

(a) Antigen - Antibody Test. The results of the antigen - antibody test are shown in Figs. 16-24. For discussion purposes only, precipitin lines were designated a, b, c, and d as shown in Fig. 16. In this test when As - red or As - iso, respectively was placed in the peripheral wells, while red - Ag or iso - Ag, respectively was placed in the central well, the expected complete fusion between the precipitin lines was observed (Figs. 17 and 18).

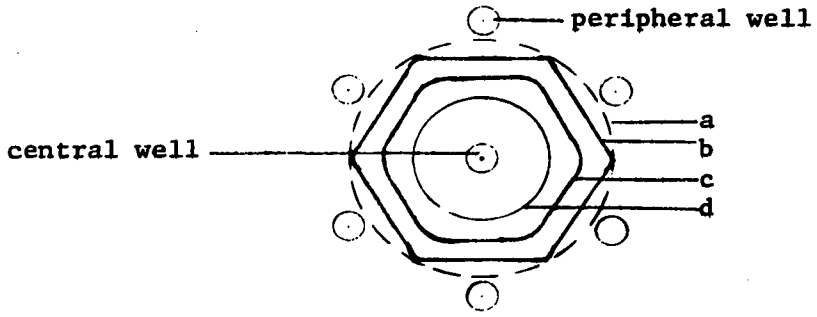


Fig. 16. The general pattern obtained in antigen - antibody test. Precipitin lines are designated as 'a', 'b', 'c', and 'd'.

It was observed that precipitin line 'a' was diffuse in Fig. 17,

but in Fig. 18 it was sharp in outline. Also, precipitin line 'c' was sharp in Fig. 17 and diffuse in Fig. 18. When red - Ag or iso - Ag, respectively was put in the peripheral wells and $(NH_4)_2SO_4$ - concentrated antiserum (conc.) was put in the central well, continuous arcs were formed as expected (Figs. 19 and 20). It was also noticed that all of the precipitin lines resulting from the red - Ag/conc. As - red reaction (Fig. 19) were more diffuse than in the reaction between iso - Ag/conc. As - red (Fig. 20). Figs. 17-20 were considered to be controls for the antigen - antibody test.

In this antigen - antibody test, no intercrossing of precipitin lines in the zone of overlap was observed (Figs. 17-24). When alternate peripheral wells contained red - Ag and iso - Ag, respectively and either As - red or As - iso was put in the central well, a complete fusion at the precipitin lines in the zone at overlap occurred, resulting in continuous arcs (Figs. 21 and 22). These results were similar to those of the controls (Figs. 17-20). Also the precipitin line 'c' was more diffuse (Fig. 22) than the corresponding 'c' line in Fig. 21 and precipitin line 'a' was sharp as shown in Fig. 22, but not in Fig. 21. As well, the distance between 'b' and 'd' precipitin lines were closer when iso - Ag reacted with As - red or As - iso, than when red - Ag reacted with As - red or As - iso (Figs. 21 and 22). When concentrated As - red or As - iso, respectively placed in the central well while the peripheral wells contained red - Ag or iso - Ag, respectively, continuous arcs were also resulted (Figs. 23 and 24). In all cases when $(NH_4)_2SO_4$ concentrated serum was used, thicker precipitin lines were formed, and precipitin line 'a' was not evident (Figs. 19, 20, 23 and 24).

Figs. 17-24. The results of the antigen - antibody test.

17. As - red placed in the peripheral wells and red- Ag was put in the central well

18. As - iso placed in the peripheral wells and iso - Ag was put in the central well

19. red - Ag placed in the peripheral wells and concentrated As - red was put in the central well

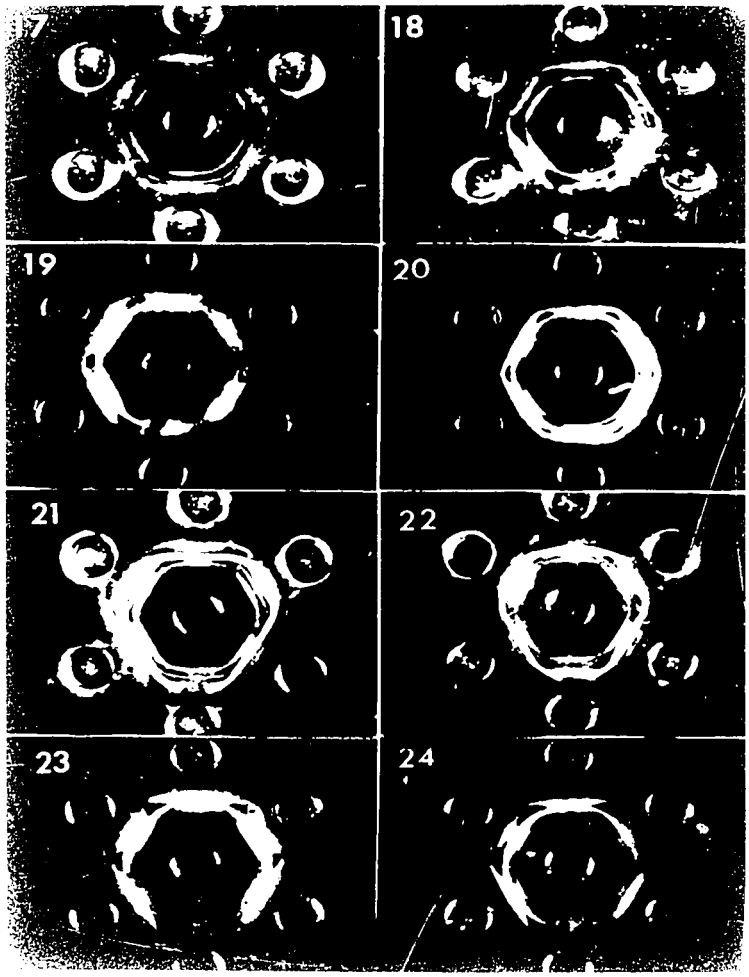
20. iso - Ag placed in the peripheral wells and concentrated As - iso was put in the central well

21. red - Ag and iso - Ag alternatively placed in the peripheral wells and As - red was put in the central well

22. red - Ag and iso - Ag alternatively placed in the peripheral wells and As - iso was put in the central well

23. red - Ag and iso - Ag alternatively placed in the peripheral wells and concentrated As - red was put in the central well

24. red - Ag and iso - Ag alternatively placed in the peripheral wells and concentrated As - iso was put in the central well.


18
19
20
21
22
23
24

(b) Immunochemical Absorption Test. Results of the immunochemical absorption test are shown in Figs. 25-30. It was found necessary to dilute the antisera 1:1 with buffered saline to ensure the precipitation of most of the antibodies when mixed with the red - Ag or iso - Ag. When aliquots of mixtures containing various ratios of, As - red to red - Ag or, As - iso to iso - Ag were placed in the peripheral wells while the central well contained red - Ag or iso - Ag, respectively, fewer precipitin lines were formed than when only As - red or As - iso, respectively were placed in the peripheral well (uppermost peripheral well in Figs. 25 and 28). It can be seen in Fig. 25 the resulting precipitin lines formed a continuous arc indicating that the antibodies had not precipitated completely even in the case of 1:1 antiserum to antigen mixture; whereas, a less evident but continuous arc was formed as shown in Fig. 28. Precipitin lines formed when either As - red was mixed with iso - Ag (Fig. 26) or when As - iso was mixed with red - Ag (Fig. 27) and aliquots of these mixtures were placed in the peripheral wells while red - Ag or iso - Ag, respectively put in the central well, differing from those of Figs. 25 and 28 were not observed. The reaction between aliquots of As - red ÷ iso - Ag and As - iso ÷ red - Ag, respectively placed in the peripheral wells and aliquots of iso - Ag or red - Ag respectively placed in the central wells are presented in Figs. 27 and 30, respectively. In these reactions (Fig. 27 and 30) a continuous arc was observed similar to that observed in Figs. 25, 28, 26, and 29 indicating that the non-precipitated antibodies in As - red or As - iso reacted in a similar manner with the antigens of the same flax variety or the antigens of the other flax variety.

Figs. 25-30. The results of the Immunochemical Absorption Test

In Figs. 25-27 and 28-30, the top peripheral well was filled with As - red or As - iso, respectively; whereas, in all Figs. 25-30 the other 5 peripheral wells were filled, clockwise, with an antiserum - antigen mixture (as indicated in each figure) of the ratios 5:1, 4:1, 3:1, 2:1 and 1:1 respectively.

25. As - red ÷ red - Ag mixture was placed in the peripheral wells and red - Ag was put in the central well

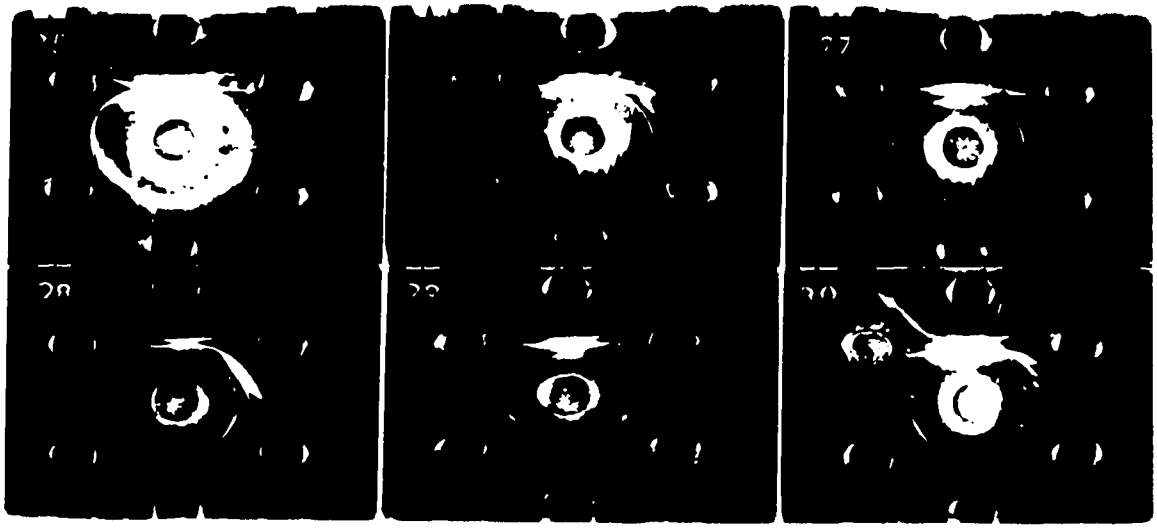
26. As - red ÷ iso - Ag mixture was placed in the peripheral wells and red - Ag was put in the central well

27. As - red ÷ iso - Ag mixture was placed in the peripheral wells and iso - Ag was put in the central well

28. As - iso ÷ iso - Ag mixture was placed in the peripheral wells and iso - Ag was put in the central well

29. As - iso ÷ red - Ag mixture was placed in the peripheral wells and iso - Ag was put in the central well

30. As - iso ÷ red - Ag mixture was placed in the peripheral wells and red - Ag was put in the central well.



4. Protein and Enzyme Studies

(a) Protein

(1) Protein determinations: The results of total protein determinations (mg protein/g fresh weight of cotyledons) in 'A_1'* and 'B'* prepared from healthy cotyledons of 9-, 15-, 18- and 24-day-old seedlings of Redwing (susceptible) and S-66-53 (immune) are shown in Table 2. Each value is the average of two determinations. Also included in Table 2 are the percentages of 'B' to that of 'A_1'.

The total protein concentration in 'A_1' and 'B' prepared from healthy cotyledons (Table 2) decreased with age of seedlings in both flax varieties. This decrease in total protein was slightly more evident for Redwing than for S-66-53.

The results for total protein concentrations (mg protein/g fresh weight of cotyledons) of 'A_2' and 'B' for non-inoculated and inoculated**cotyledons of Redwing (susceptible) and S-66-53 (immune) seedlings are shown in Table 3. Each value is the average of two determinations.

The total protein concentration of 'A_2' as shown in Table 3, of non-inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53 respectively were considerably lower than those of healthy cotyledons of 18-day-old seedlings as shown in Table 2. After inoculation (Table 3), the total protein concentrations were slightly higher in inoculated cotyledons than in the corresponding controls. The total protein concentration in 'B' of Redwing and S-66-53 decreased slightly

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* See Material and Methods for the designation of the various extracts.

** Inoculations made when seedlings were 9 days old.

Table 2

Total protein concentration (mg protein/g fresh weight of cotyledons) in the crude extracts ('A_1') and 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Total protein conc. of 'A_1' mg/g of fresh weight		Total protein conc. of 'B' mg/g of fresh weight		Yield* %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9	9.63	9.88	0.85	1.32	8.85	13.34
15	8.52	8.22	1.15	1.09	13.47	13.22
18	6.57	8.03	0.90	0.95	13.75	11.78
24	6.21	8.03	0.76	0.84	12.21	10.48

* $\frac{\text{Total protein of 'B' x 100}}{\text{'}A_1\text{'}}$

Table 3

Total protein (mg/g fresh weight of cotyledons) in the dialyzed crude extracts 'A_2' and 50 - 90% $(NH_4)_2SO_4$ - precipitated fractions ('B') from non-inoculated and inoculated cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Total protein conc. of 'A_2' mg/g of fresh weight		Total protein conc. of 'B' mg/g of fresh weight		Yield* %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9(non-inoculated)	10.15	9.97	1.14	0.69	11.23	6.93
18(non-inoculated)	3.08	3.52	0.70	0.65	22.60	18.55
18(inoculated)	3.56	4.53	1.00	0.88	28.07	19.50

* $\frac{\text{Total protein of 'B'} \times 100}{\text{Total protein of '}A_2\text{'}}$

with age of seedlings in agreement with data shown in Table 2. Inoculated cotyledons of Redwing and S-66-53 had a higher protein concentration in 'B' than corresponding controls.

Total protein concentration in 'A_2' and extract 'B' for the non-inoculated cotyledons of 18-day-old Redwing and S-66-53 seedlings (Table 3) were considerably lower than those of healthy cotyledons of 18-day-old seedlings (Table 2).

(2) Electrophoretic patterns of protein bands: In the preliminary experiments a larger number of more distinct bands were obtained for 'B' than for 'A_1' or 'A_2'.

The electrophoretic patterns of protein bands of the various extracts were similar (Figs. 31-34). A typical scan for the electrophoretic pattern of protein bands is shown in Fig. 35.

Age of cotyledons, and inoculation with rust fungus did not seem to affect the patterns of protein bands in both varieties.

Protein bands that were observed in one variety but not in the other were marked with arrows in the figures. Bands observed either in the non-inoculated or the inoculated cotyledons of each variety were marked with (*).

(b) Enzyme Studies

(1) Peroxidase:

(a) Peroxidase activities. The results of peroxidase specific activities (units/mg of protein) in 'A_1' and 'B' prepared from healthy cotyledons of 9-, 15-, 18- and 24-day-old seedlings of Redwing and S-66-53 are shown in Table 4. Each value is the average of two assays. Also, percentage increase of activity in 'B' over the activity in 'A_1' are included in Table 4 to indicate changes in peroxidase speci-

Fig. 31. Electrophoretic patterns of protein bands of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other.

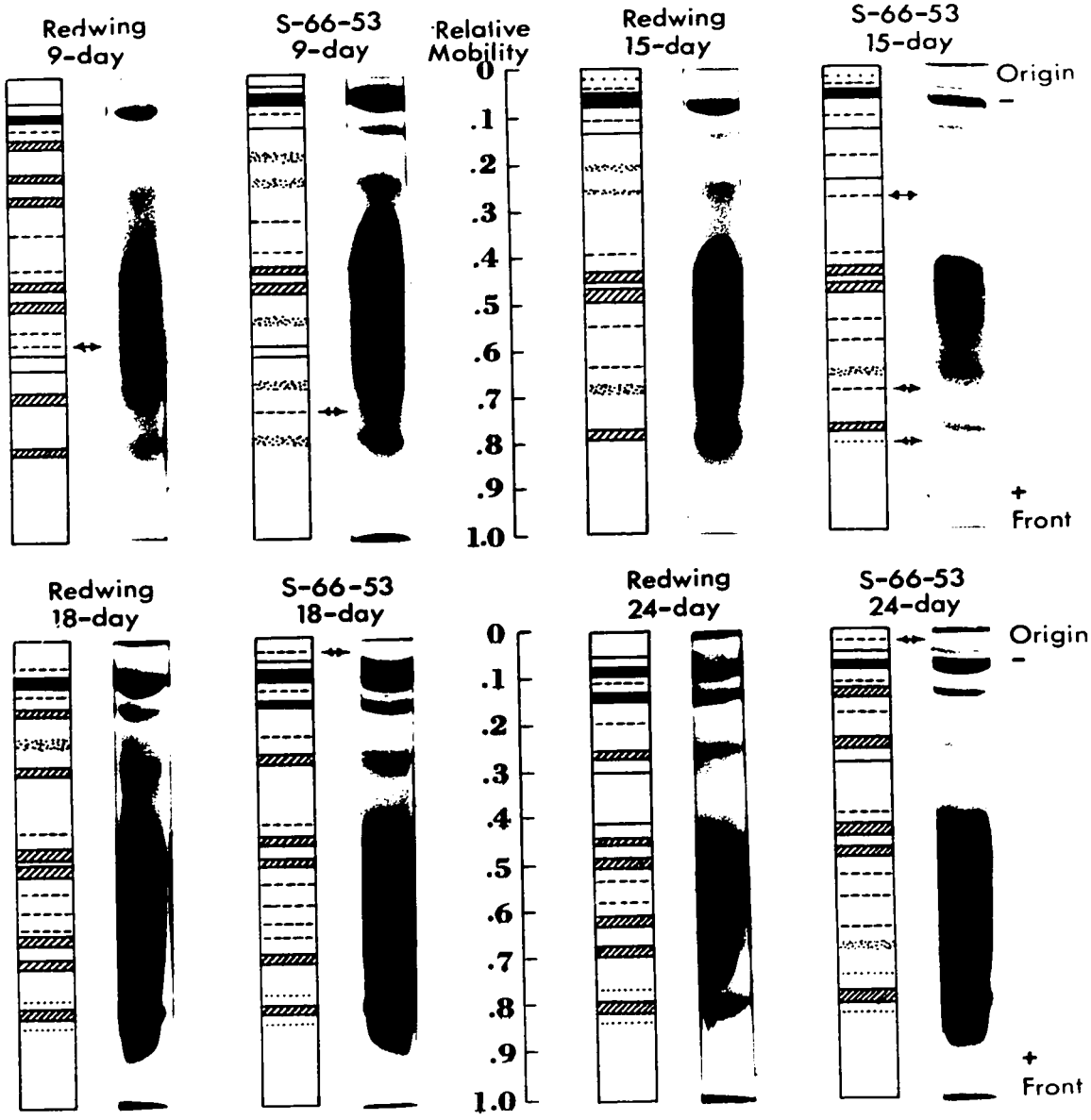

Redwing
9-day
S-66-53
9-day
Relative
Mobility
Redwing
15-day
S-66-53
15-day
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
S-66-53
18-day
Redwing
24-day
S-66-53
24-day
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front

Fig. 32. Electrophoretic patterns of protein bands of Red-Ag and iso-Ag (90% $(NH_4)_2SO_4$ - precipitated protein fractions) of 15-day-old seedlings of Redwing and S-66-53.

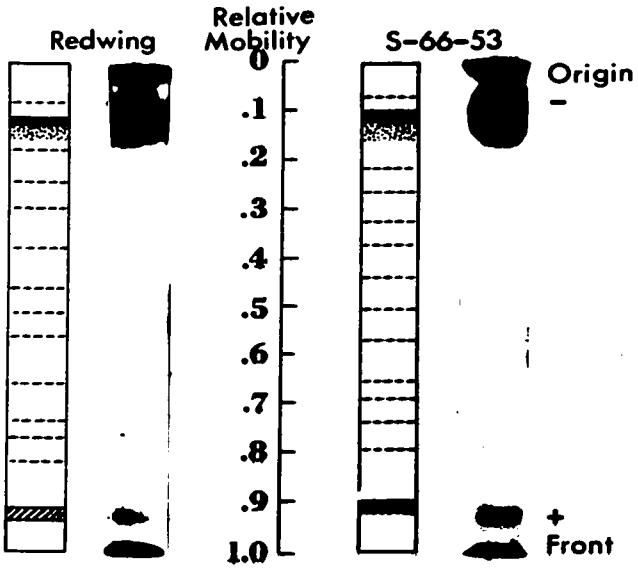

Redwing
Relative Mobility
S-66-53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
−
+
Front

Fig. 33. Electrophoretic patterns of protein bands of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated cotyledons of 9-, and 18-day-old seedlings, and inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other. The * bands were observed in either the non-inoculated or in inoculated cotyledons of each variety.

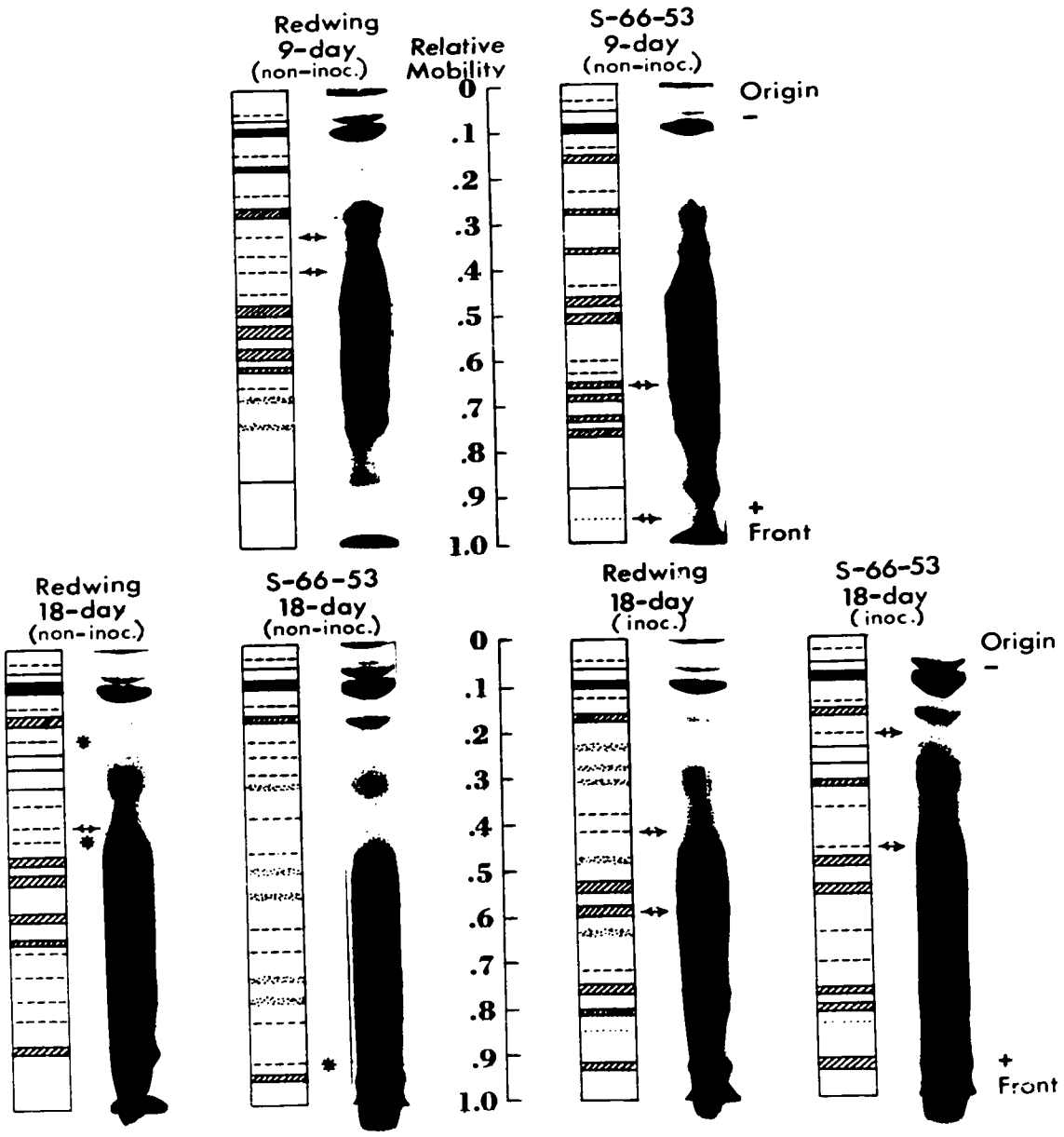

Redwing
9-day
(non-inoc.)
Relative
Mobility
S-66-53
9-day
(non-inoc.)
Origin
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
(non-inoc.)
S-66-53
18-day
(non-inoc.)
Redwing
18-day
(inoc.)
S-66-53
18-day
(inoc.)
Origin
+
Front

Fig. 34. Electrophoretic patterns of protein bands of dialyzed crude extracts ('A_2') from inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53.

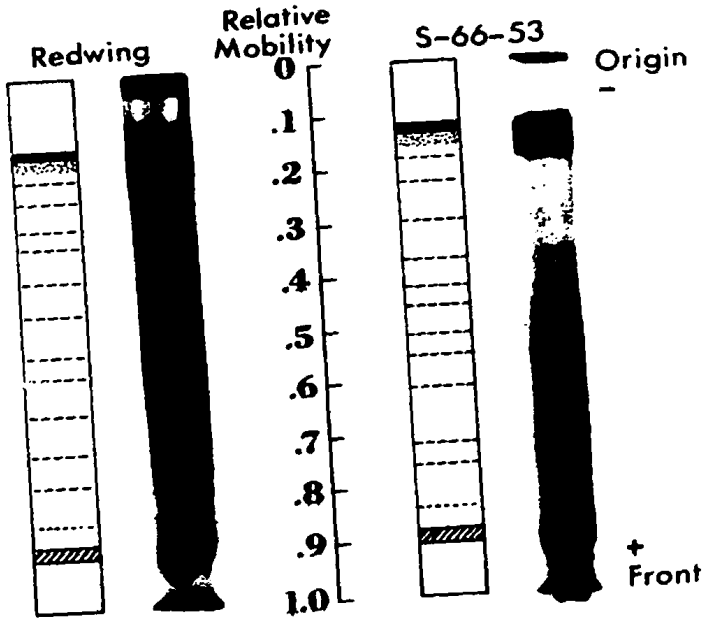

Redwing
Relative Mobility
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
S-66-53
Origin
−
+
Front

Fig. 35. Typical scan for the electrophoretic pattern of protein bands.

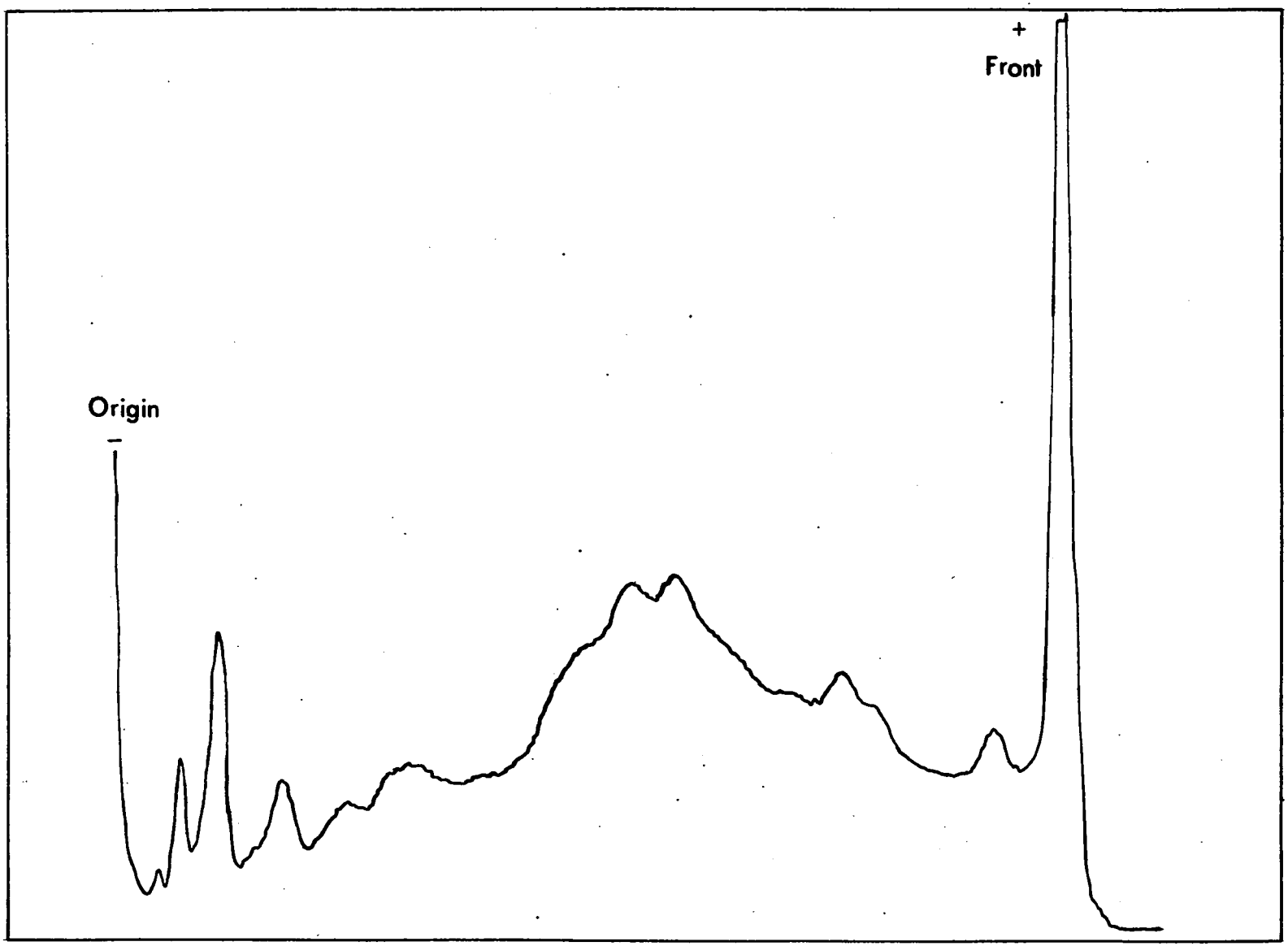

+
Front
Origin
–

Table 4

Peroxidase specific activities (units/mg of protein) in the crude extracts ('A_1') and in the 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy flax cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Peroxidase specific activity of 'A_1' units/mg of protein		Peroxidase specific activity of 'B' units/mg of protein		Yield* %		**Increase of specific activity as a result of fractionation %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9	0.79	1.45	7.65	6.81	86.14	50.33	973.34	469.40
15	2.30	1.70	12.45	11.88	72.85	92.15	540.77	696.91
18	4.22	2.89	15.55	16.12	50.65	65.93	368.42	557.99
24	5.12	3.90	23.41	19.30	55.88	51.91	457.55	459.12

* $\%\ \text{yield} = \frac{\text{total activity of 'B'} \times 100}{\text{total activity of '}A_1\text{'}}$

** $\frac{\text{Peroxidase specific activity of 'B'} \times 100}{\text{Peroxidase specific activity of '}A_1\text{'}}$

fic activities as a result of fractionation with ammonium sulfate.

Peroxidase specific activities in 'A_1' as well as in 'B' increased with age for healthy cotyledons of Redwing and S-66-53 seedlings (Table 4). Peroxidase activities were slightly higher in 'A_1' of Redwing than for that of S-66-53. Peroxidase specific activities were much higher in 'B' than in 'A_1'. 'A_1' of Redwing had slightly higher peroxidase specific activities except for cotyledons of 18-day-old seedlings in which slightly lower activity than in the corresponding cotyledons of S-66-53 was observed.

The peroxidase specific activities (units/mg of protein) of 'A_2' and 'B' for non-inoculated and inoculated cotyledons of Redwing and S-66-53 seedlings are shown in Table 5. Each value is the average of two assays. Percent yield of 'B' to that of 'A_2' is included in the Table. Also included in Table 5 is the percentage increase of specific activity in 'B' over the specific activity in 'A_2' to indicate changes in peroxidase activity as a result of fractionation with ammonium sulfate.

Peroxidase specific activities of 'B' prepared from non-inoculated cotyledons of 18-day-old seedlings of each flax variety were much higher than those of 9-day-old seedlings (Table 5), in agreement with the data in Table 4, which indicates that peroxidase activity had increased with the age of cotyledons. In 'A_2' of non-inoculated and inoculated cotyledons of 18-day-old seedlings peroxidase specific activities were slightly higher in Redwing than in S-66-53, however, inoculated cotyledons of each variety have lower peroxidase activity than corresponding controls (Table 5). 'B' showed much higher peroxidase specific activities than those of 'A_2'. After inoculation, peroxidase activities

Table 5

Peroxidase specific activities (units/mg of protein) in the dialyzed crude extracts ('A'_2) and in the 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated (control) and inoculated flax cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Peroxidase specific activity of 'A_2' units/mg of protein		Peroxidase specific activity of 'B' units/mg of protein		Yield* %		**Increase of specific activity as a result of fractionation %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9(non-inoculated)	1.05	1.03	6.00	5.08	64.22	34.16	571.65	429.92
18(non-inoculated)	6.08	5.07	19.09	11.20	70.93	40.93	313.90	220.70
18(inoculated)	5.84	4.55	12.45	13.21	59.90	56.59	213.36	290.17

* % yield = $\frac{\text{total activity of 'B' x 100}}{\text{total activity of '}A_2\text{'}}$

** $\frac{\text{Peroxidase specific activity of 'B' x 100}}{\text{Peroxidase specific activity of '}A_2\text{'}}$

in 'B' decreased in Redwing and slightly increased in S-66-53 than in corresponding controls.

(b) Electrophoretic patterns of peroxidase isoenzymes. The electrophoretic patterns of peroxidase isoenzyme bands of the various extracts did not differ significantly (Figs. 36-39). A typical scan for the electrophoretic patterns of peroxidase isoenzymes is shown in Fig. 40.

As in proteins, age of cotyledons and inoculation with rust fungus did not seem to affect the pattern of peroxidase isoenzymes in both varieties.

Any isoenzyme band that was observed in one variety but not in the other is marked with arrow in the figures. Isoenzyme bands observed either in the non-inoculated or the inoculated cotyledons of each variety are marked with (*).

(2) Polyphenol oxidase(s):

(a) Polyphenol oxidase(s) activities. The results of polyphenol oxidase(s) specific activities (units/mg of protein) in 'A_2' and 'B' of non-inoculated and inoculated cotyledons of Redwing and S-66-53 are shown in Table 6. Each value is the average of two assays.

Polyphenol oxidase(s) specific activities in 'A_2' of non-inoculated cotyledons of both 9- and 18-day-old Redwing seedlings did not differ appreciably (Table 6). However, in non-inoculated S-66-53 polyphenol oxidase(s) activity increased with age of cotyledons. Upon inoculation polyphenol oxidase(s) activities in both varieties decreased considerably; however, the decrease in Redwing was greater than in S-66-53. Polyphenol oxidase(s) activities for the non-inoculated and inoculated cotyledons of S-66-53 seedlings were higher than those of Redwing.

In 'B' of Redwing, polyphenol oxidase(s) specific activity incr-

Fig. 36. Electrophoretic patterns of peroxidase isoenzymes of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of Redwing and S-66-53. The ⟷ bands were observed in one variety but not in the other.

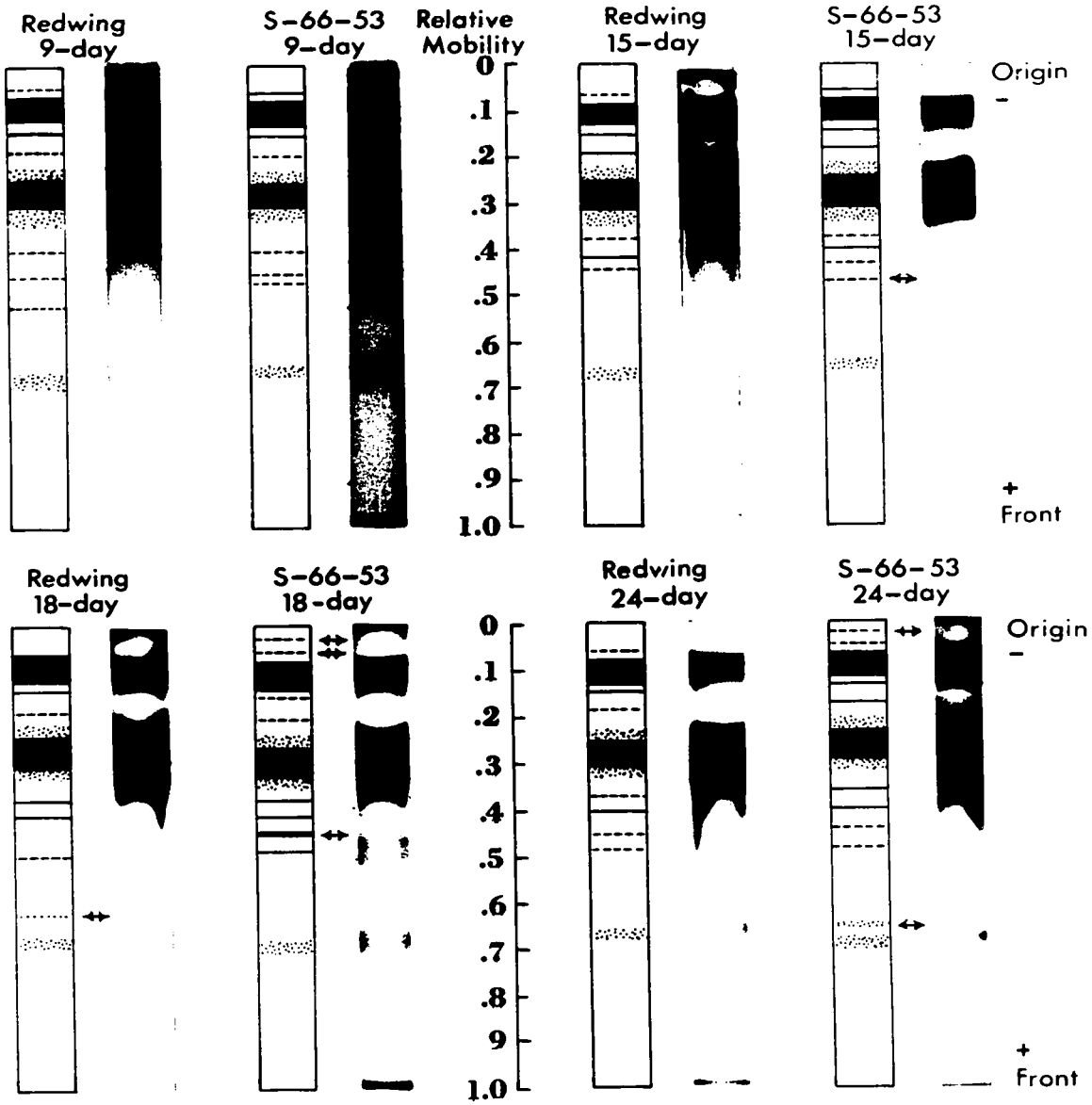

Redwing
9-day
S-66-53
9-day
Relative
Mobility
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Redwing
15-day
S-66-53
15-day
Origin
−
+
Front
Redwing
18-day
S-66-53
18-day
Redwing
24-day
S-66-53
24-day
Origin
−
+
Front

Fig. 37. Electrophoretic patterns of peroxidase isoenzymes of Red-Ag and iso-Ag (90% $(NH_4)_2SO_4$ - precipitated protein fractions) of 15-day-old seedlings of Redwing and S-66-53.

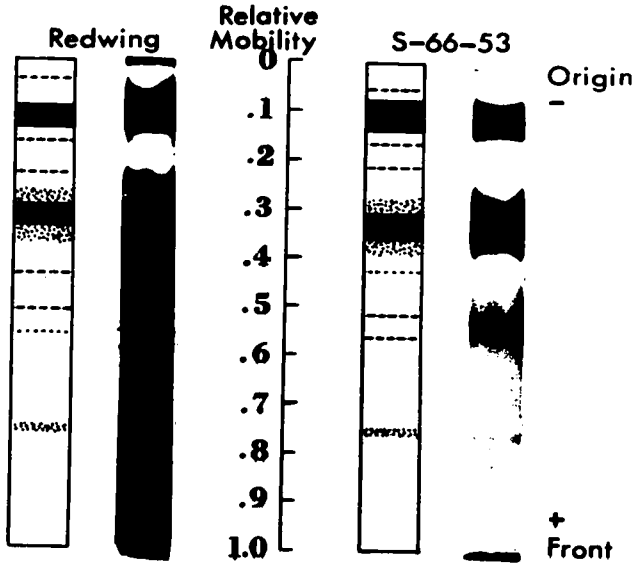

Redwing
Relative Mobility
S-66-53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
−
+
Front

Fig. 38. Electrophoretic patterns of peroxidase isoenzymes of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated cotyledons of 9-, and 18-day-old seedlings, and inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other. The * bands were observed in either the non-inoculated or the inoculated cotyledons of each variety.

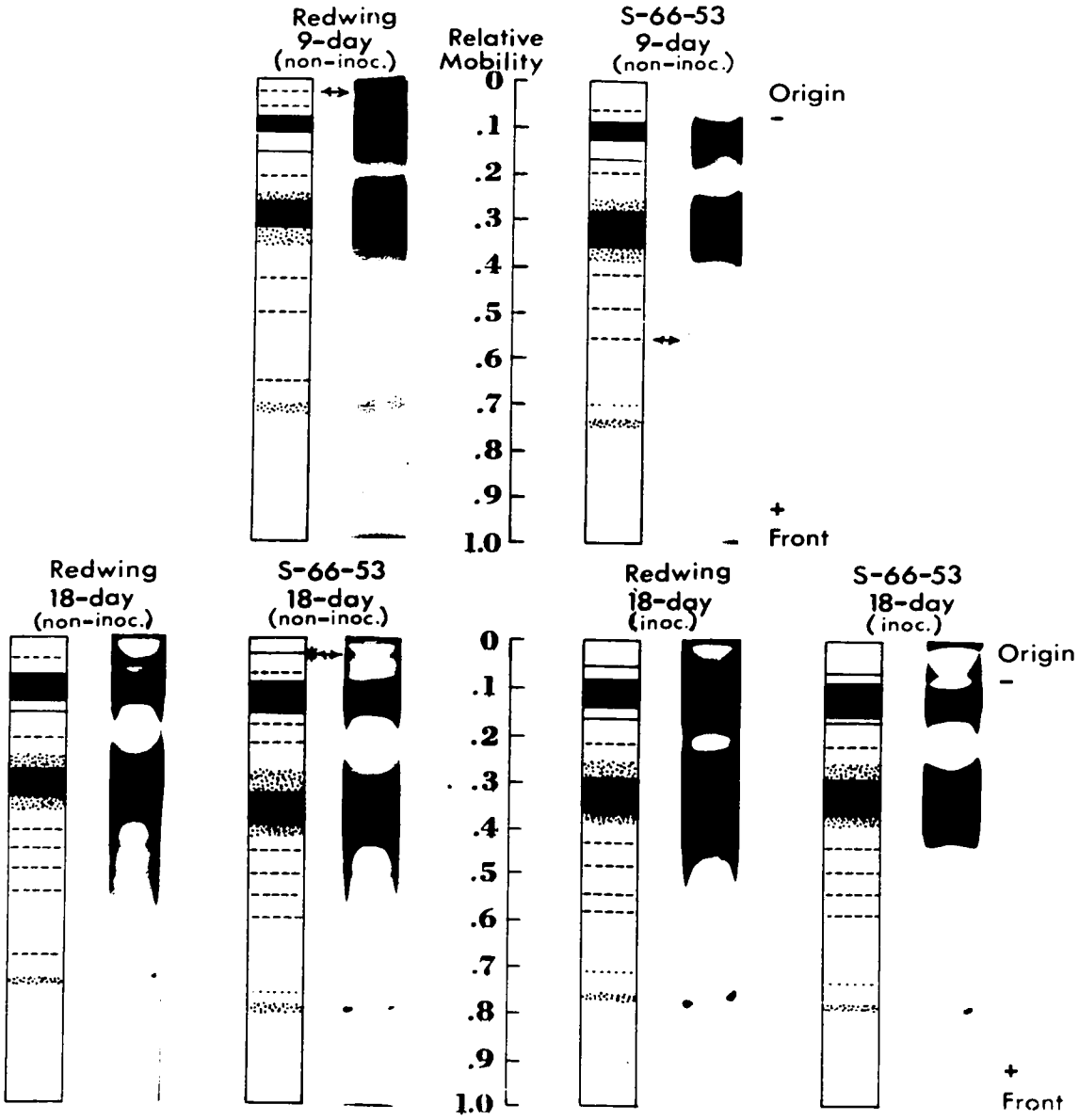

Redwing
9-day
(non-inoc.)
Relative
Mobility
S-66-53
9-day
(non-inoc.)
Origin
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
(non-inoc.)
S-66-53
18-day
(non-inoc.)
Redwing
18-day
(inoc.)
S-66-53
18-day
(inoc.)
Origin
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front

Fig. 39. Electrophoretic patterns of peroxidase isoenzymes of dialyzed crude extracts ('A_2') from inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other.

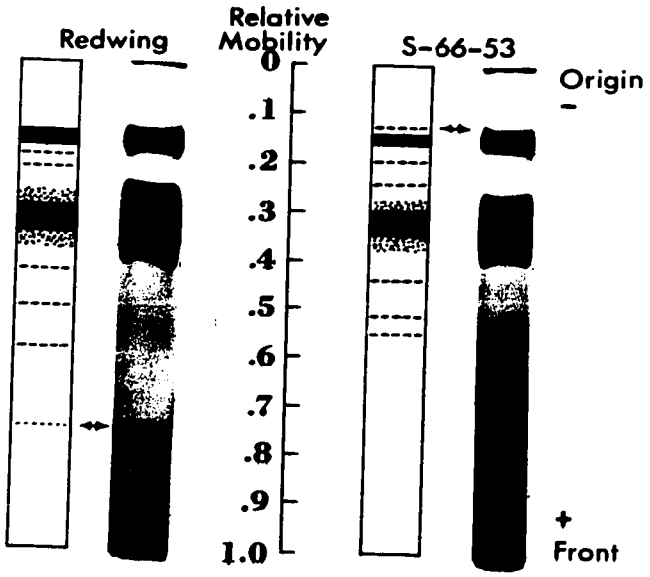

Redwing
Relative Mobility
S-66-53
Origin
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front

Fig. 40. Typical scan for the electrophoretic pattern of peroxidase isoenzymes.

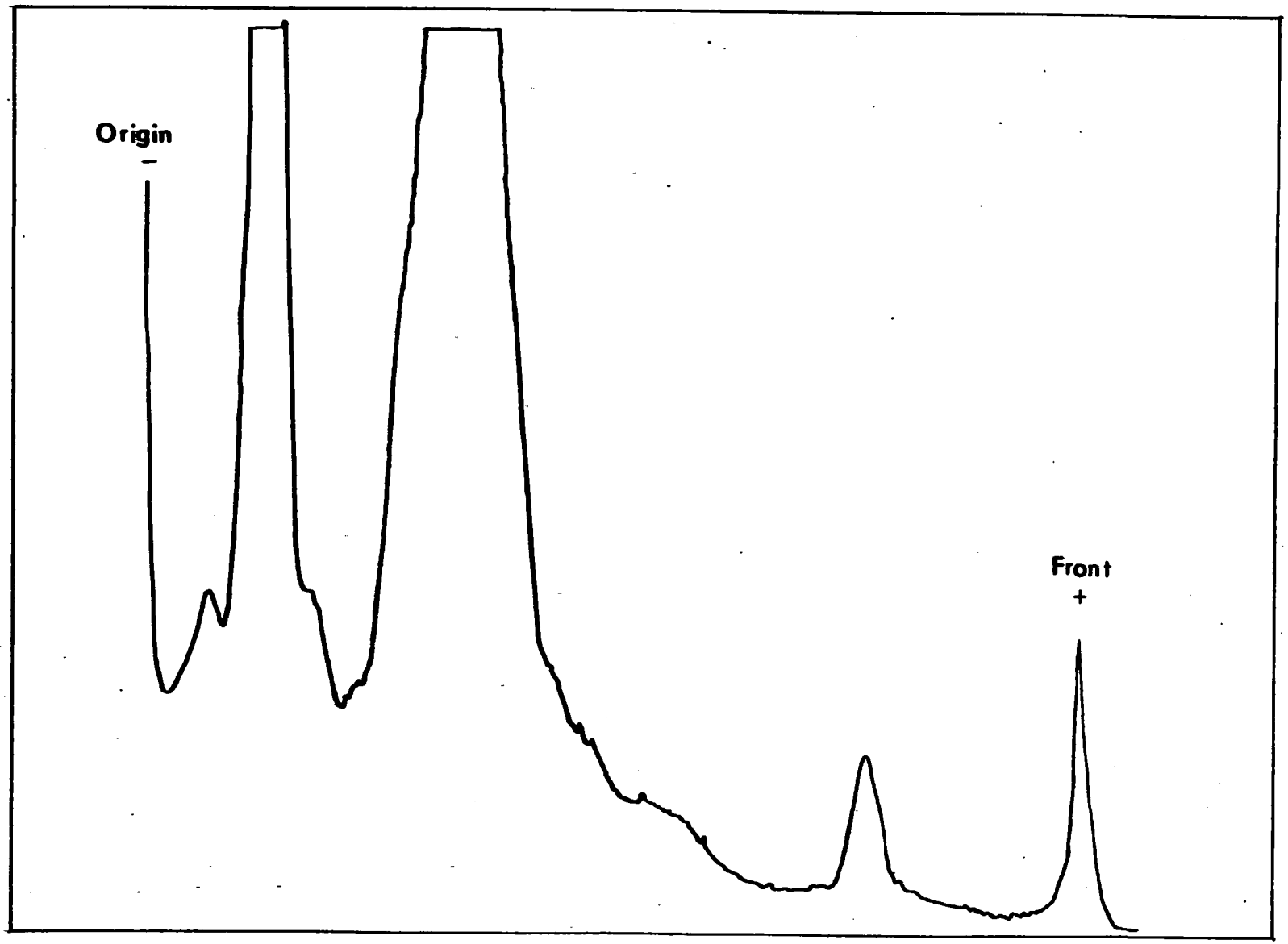

Origin
−
Front
+

Table 6

Polyphenol oxidase(s) specific activities (units/mg of protein) in the dialyzed crude extracts ('A_2') and 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated and inoculated cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Polyphenol oxidase(s) specific activity in 'A_2' units/mg of protein		Polyphenol oxidase(s) specific activity in 'B' units/mg of protein		Yield* %		**Increase or decrease of specific activity as a result of fractionation %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9(non-inoculated)	0.40	0.61	0.05	0.19	1.55	2.20	13.82	31.80
18(non-inoculated)	0.40	1.08	0.18	0.18	10.03	3.04	44.38	16.38
18(inoculated)	0.07	0.30	0.08	0.02	35.30	1.54	125.75	7.88

* $\%\ \text{yield} = \frac{\text{total activity of 'B' x 100}}{\text{total activity of '}A_2\text{'}}$

** $\frac{\text{specific activity of 'B' x 100}}{\text{specific activity of '}A_2\text{'}}$

eased with age of cotyledons but decreased after inoculation. In S-66-53, polyphenol oxidase(s) specific activity was approximately the same in cotyledons of 9- and 18-day-old seedlings but decreased in the inoculated cotyledons. In cotyledons of 9-day-old seedlings, polyphenol oxidase(s) specific activity in partially purified protein fraction was higher in S-66-53. Polyphenol oxidase(s) specific activity in 'B' was approximately the same for both varieties in the cotyledons of 18-day-old seedlings, but in the inoculated cotyledons of Redwing, polyphenol oxidase(s) activity was higher than that of S-66-53 (Table 6).

(b) Electrophoretic patterns of polyphenol oxidase isoenzymes. The electrophoretic patterns of polyphenol oxidase isoenzymes of the various extracts were very similar (Figs. 41-44). A typical scan for the electrophoretic patterns of polyphenol oxidase isoenzymes is shown in Fig. 45. However, an additional isoenzyme band was observed in Fig. 42 and Fig. 43 (except for non-inoculated cotyledons of 9-day-old seedlings of S-66-53, Fig. 43).

(3) β-Glucosidase activities: The results of β-glucosidase specific activities (units/mg of protein) in 'A_1' and 'B' prepared from healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of Redwing and S-66-53 are shown in Table 7. Each value is the average of two assays.

β-glucosidase specific activities in 'A_1' as well as in 'B' increased with age of cotyledons in Redwing and S-66-53 except for the cotyledons of 24-day-old S-66-53 seedlings in which lower β-glucosidase activity was observed (Table 7). β-glucosidase specific activities generally were slightly higher in S-66-53 than in Redwing. β-glucosidase specific activities in 'B' were much higher than in those of 'A_1'.

Fig. 41. Electrophoretic patterns of polyphenol oxidase(s) isoenzymes of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of Redwing and S-66-53.

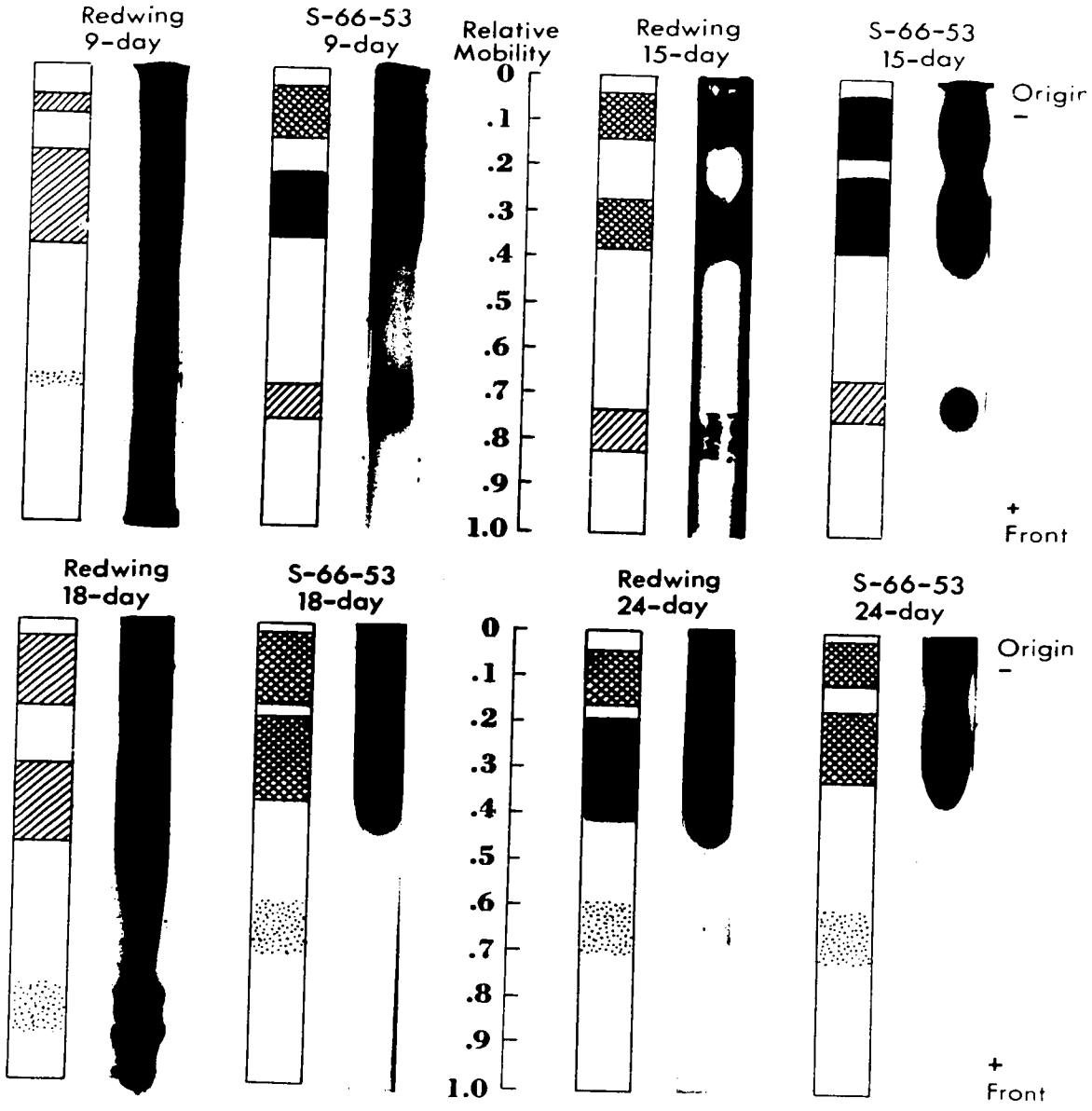

Redwing
9-day
S-66-53
9-day
Relative
Mobility
Redwing
15-day
S-66-53
15-day
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
S-66-53
18-day
Redwing
24-day
S-66-53
24-day
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front

Fig. 42. Electrophoretic patterns of polyphenol oxidase(s) isoenzymes of Red-Ag and iso-Ag (90% $(NH_4)_2SO_4$ - precipitated protein fractions) of 15-day-old seedlings of Redwing and S-66-53.

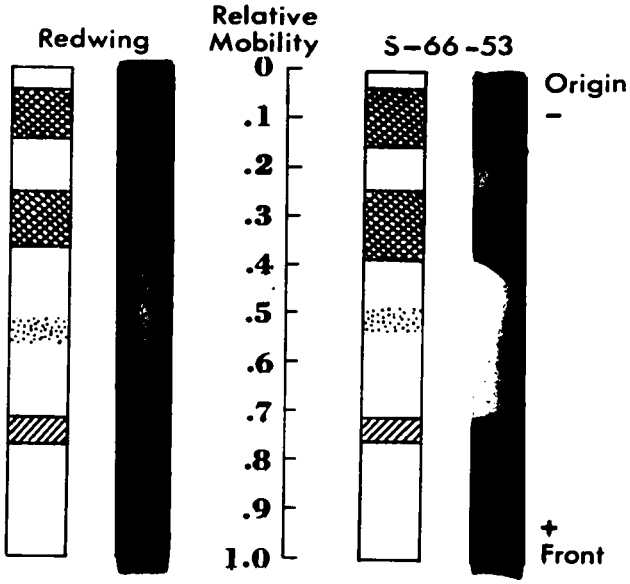

Redwing
Relative Mobility
S-66-53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
-
+
Front

Fig. 43. Electrophoretic patterns of polyphenol oxidase(s) isoenzymes of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated cotyledons of 9-, and 18-day-old seedlings, and inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other.

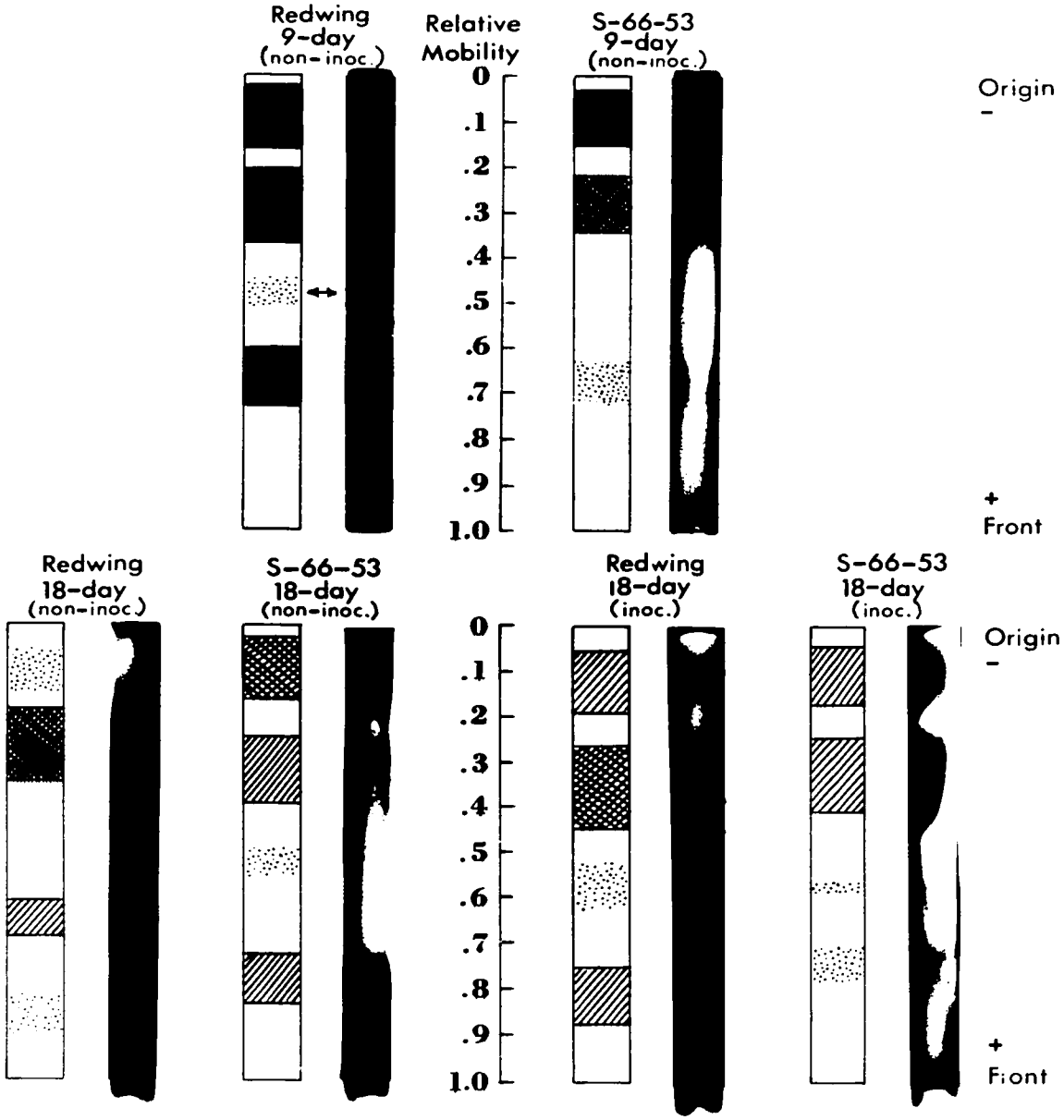

Redwing
9-day
(non-inoc.)
Relative
Mobility
S-66-53
9-day
(non-inoc.)
Origin
-
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
(non-inoc.)
S-66-53
18-day
(non-inoc.)
Redwing
18-day
(inoc.)
S-66-53
18-day
(inoc.)
Origin
-
+
Front

Fig. 44. Electrophoretic patterns of polyphenol oxidase(s) of dialyzed crude extracts ('A_2') from inoculated cotyledons of 18-day old seedlings of Redwing and S-66-53.

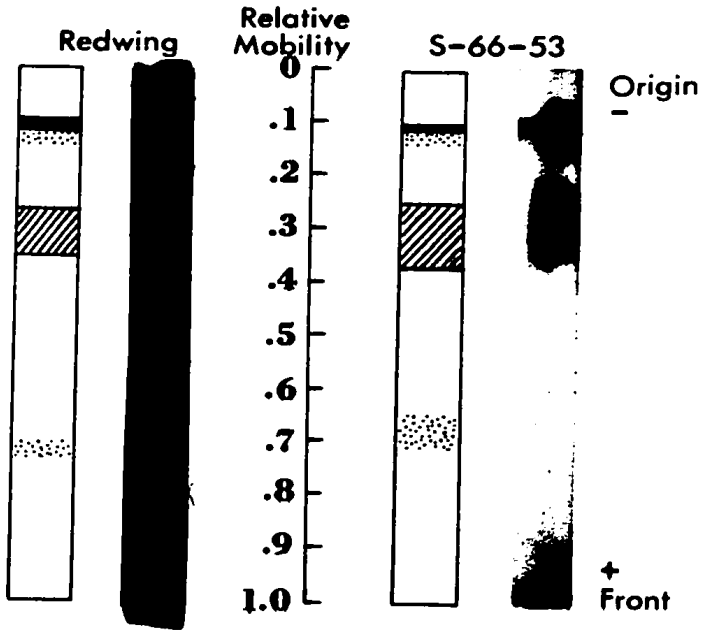

Redwing
Relative Mobility
S-66-53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
−
+
Front

Fig. 45. Typical scan for the electrophoretic pattern of polyphenol oxidase isoenzymes.

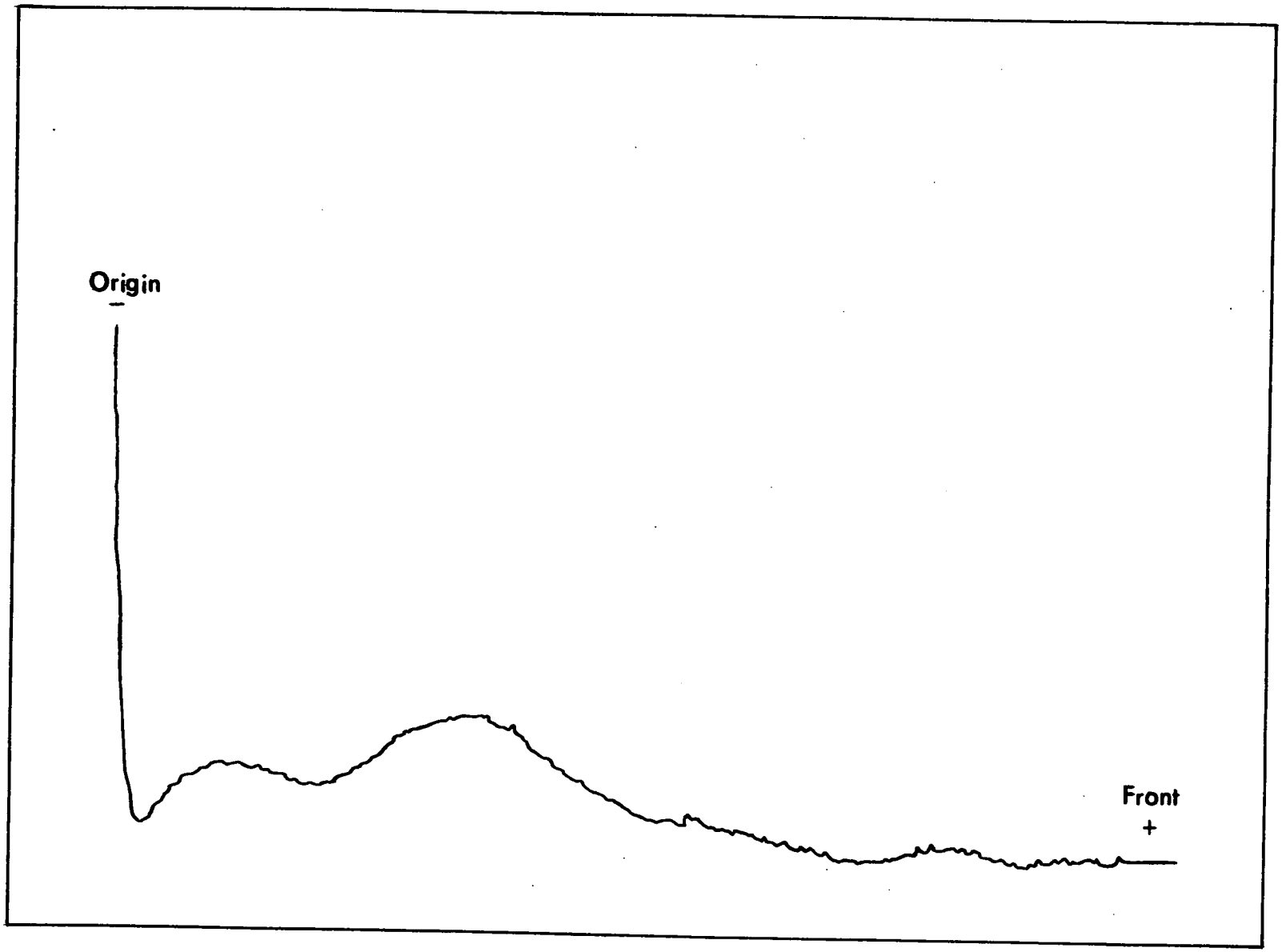

Origin
−
Front
+

Table 7

β-Glucosidase specific activities (units/mg of protein) in the crude extracts ('A_1') and in the 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy flax cotyledons of Redwing and S-66-53.

Age of Seedlings (days)	β-glucosidase specific activity of 'A_1' units/mg of protein		β-glucosidase specific activity of 'B' units/mg of protein		Yield* %		**Increase of specific activity as a result of fractionation %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9	0.30	0.52	1.79	2.52	57.07	64.86	589.41	486.07
15	0.43	0.54	1.99	2.61	59.19	63.62	460.22	481.14
18	0.49	0.63	2.24	2.72	62.70	51.38	456.10	434.85
24	0.58	0.50	2.45	2.31	51.18	48.64	419.05	463.91

* $\%\ \text{yield} = \frac{\text{total activity of 'B'} \times 100}{\text{total activity of '}A_1\text{'}}$

** $\frac{\text{specific activity of 'B'} \times 100}{\text{specific activity of '}A_1\text{'}}$

β-Glucosidase specific activities of 'A_2' and 'B' for non-inoculated and inoculated cotyledons of Redwing and S-66-53 seedlings are shown in Table 8. Each value is the average of two assays.

In 'A_2' of non-inoculated cotyledons of Redwing and S-66-53, β-glucosidase specific activities (Table 8) increased with age of cotyledons. β-Glucosidase specific activity of 'A_2' for cotyledons of 9-day-old seedlings was slightly higher in S-66-53 than in Redwing; however, non-inoculated cotyledons of 18-day-old seedlings of Redwing had a slightly higher β-glucosidase specific activity than S-66-53. β-Glucosidase specific activities for both varieties were slightly lower in inoculated cotyledons than for each of the corresponding controls. Also, after inoculation β-glucosidase specific activity of Redwing was slightly higher than that of S-66-53. β-Glucosidase specific activities in 'B' were higher than those of 'A_2'. In the non-inoculated cotyledons of S-66-53 β-glucosidase specific activities for 'B' increased with age of cotyledons but not in Redwing. Inoculated cotyledons of both varieties, β-glucosidase specific activities 'B' were slightly higher than those of corresponding controls. β-Glucosidase specific activities in 'A_2' for non-inoculated cotyledons (Table 8) were higher than those for 'A_2' of healthy cotyledons of 9-, and 18-day-old seedlings of both varieties (Table 7).

(4) Electrophoretic patterns of esterase isoenzymes: The electrophoretic patterns of esterase isoenzymes of the various extracts did not differ significantly (Figs. 46-49). A typical scan for the electrophoretic patterns of esterase isoenzymes is shown in Fig. 50.

The esterase(s) band adjacent to the front band (bromophenol blue) was not observed for 18-, or 24-day-old seedlings in 'B' or Red-

Table 8

β-Glucosidase specific activities (units/mg of protein) in the dialyzed crude extracts ('A_2') and in the 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from non-inoculated and inoculated cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	β-glucosidase specific activity of 'A_2' units/mg of protein		β-glucosidase specific activity of 'B' units/mg of protein		Yield* (%)		**Increase in specific activity as a result of fractionation %	
	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53	REDWING	S-66-53
9(non-inoculated)	0.41	0.54	2.35	1.45	65.28	18.77	581.04	270.89
18(non-inoculated)	1.22	9.08	1.69	2.23	31.45	38.28	139.17	206.42
18(inoculated)	1.15	1.06	2.15	2.84	51.78	50.53	187.16	268.54

* % yield = $\frac{\text{total activity of 'B' x 100}}{\text{total activity of '}A_2\text{'}}$

** $\frac{\text{specific activity of 'B' x 100}}{\text{specific activity of '}A_2\text{'}}$

Fig. 46. Electrophoretic patterns of esterase isoenzymes of 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions ('B') from healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other.

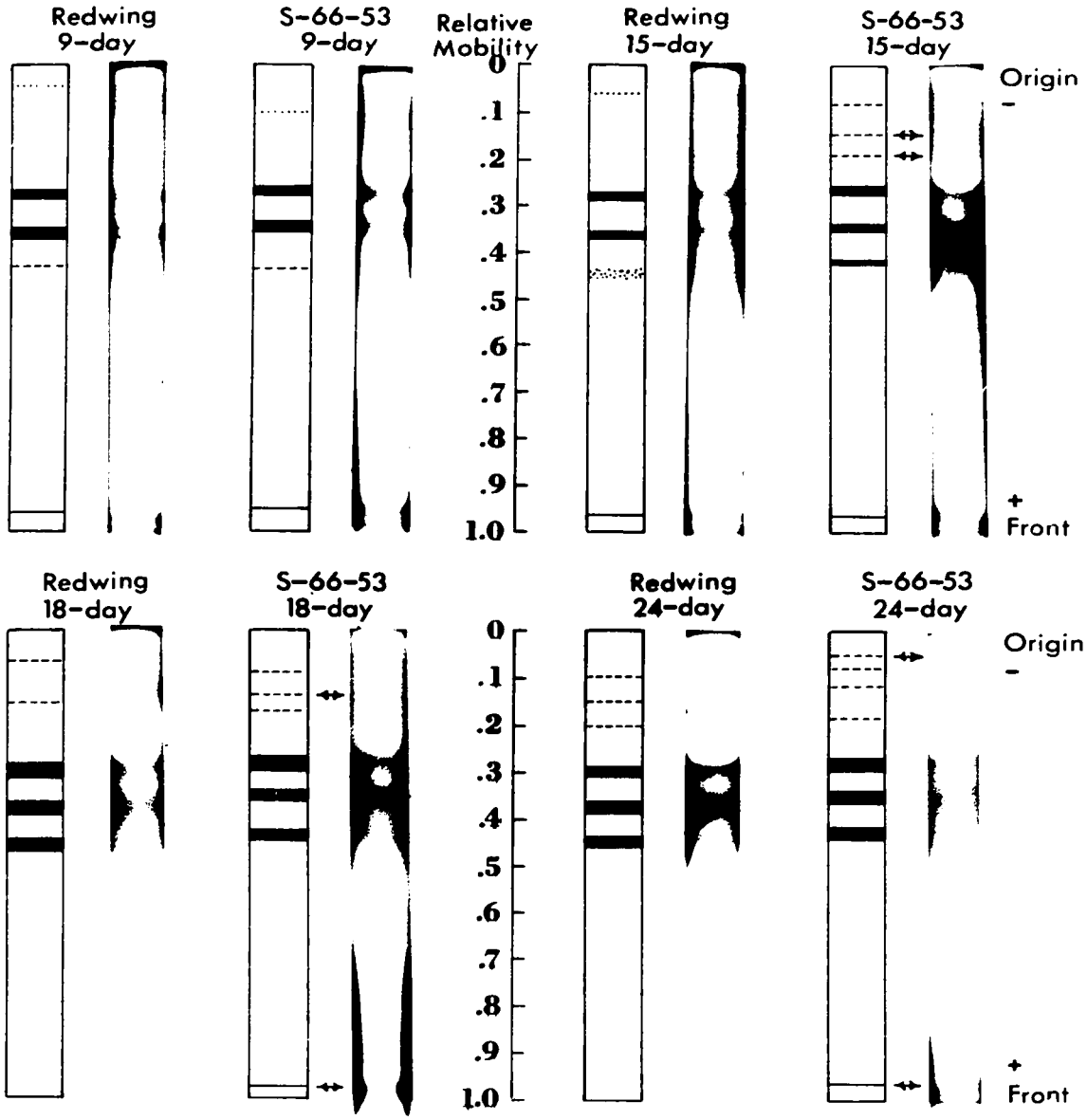

Redwing
9-day
S-66-53
9-day
Relative
Mobility
Redwing
15-day
S-66-53
15-day
Origin
-
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
S-66-53
18-day
Redwing
24-day
S-66-53
24-day
Origin
-
+
Front

Fig. 47. Electrophoretic patterns of esterase isoenzymes of Red-Ag and iso - Ag (90% $(NH_4)_2SO_4$ - precipitated protein fractions) of 15-day-old seedlings of Redwing and S-66-53.

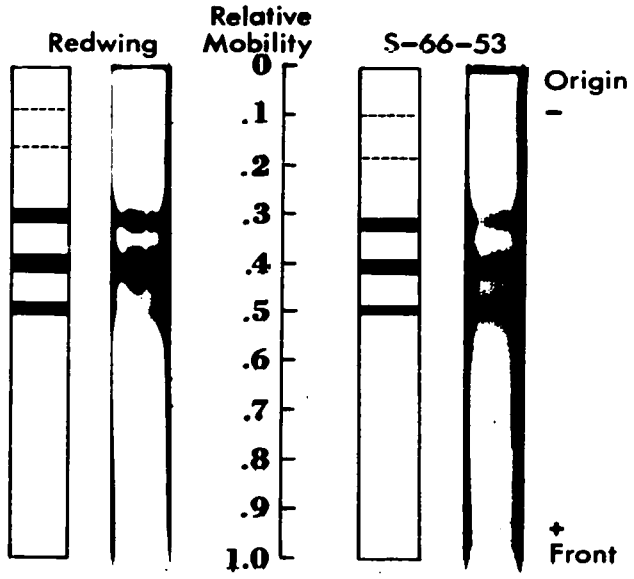

Redwing
Relative Mobility
S-66-53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
−
+
Front

Fig. 48. Electrophoretic patterns of esterase isoenzymes of 50 - 90% $(NH_4)SO_4$ - precipitated protein fractions ('B') from non-inoculated cotyledons of 9-, and 18-day-old seedlings, and inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53. The ↔ bands were observed in one variety but not in the other. The * bands were observed in either the non-inoculated or the inoculated cotyledons of each variety.

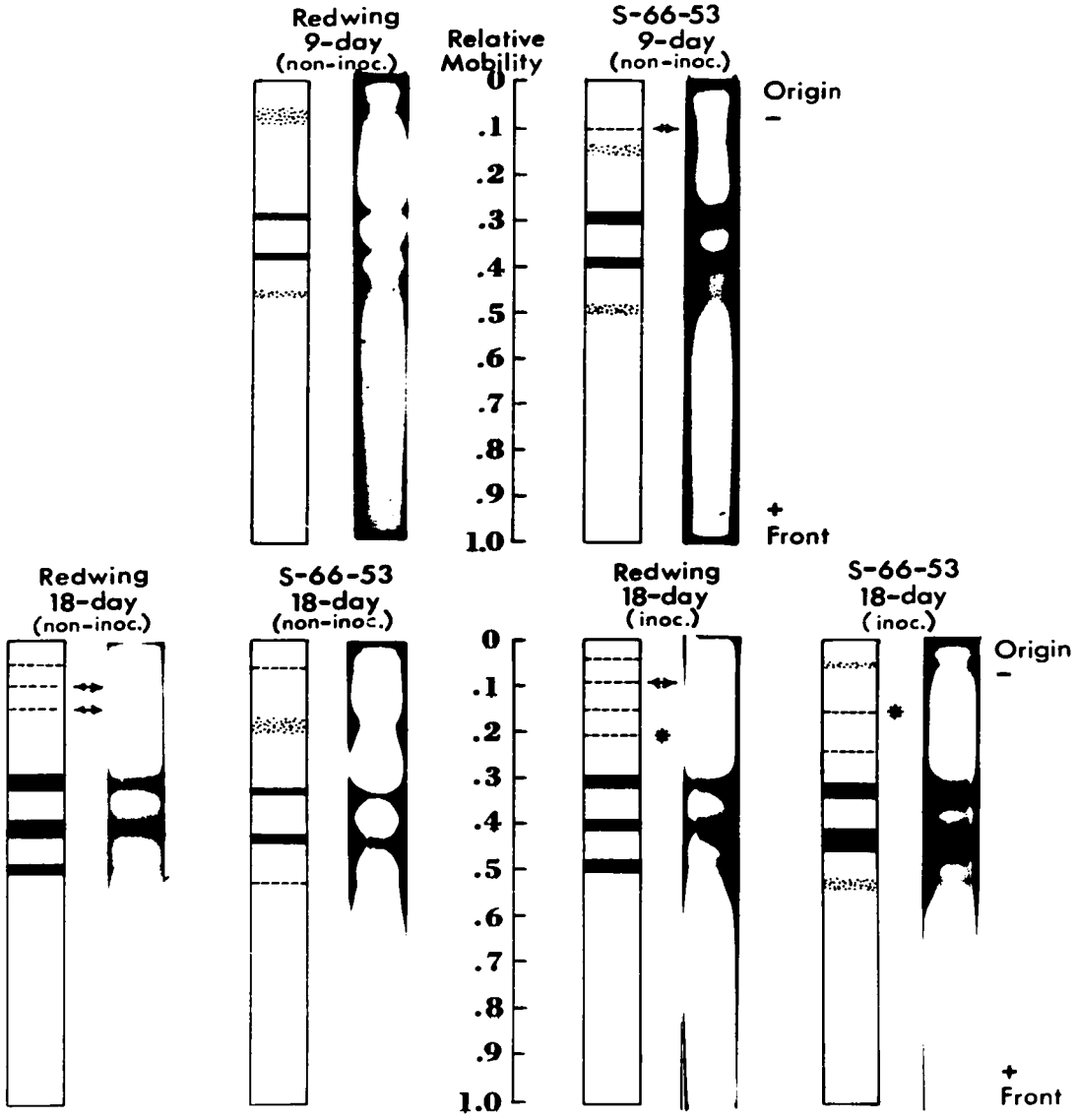

Redwing
9-day
(non-inoc.)
Relative
Mobility
S-66-53
9-day
(non-inoc.)
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front
Redwing
18-day
(non-inoc.)
S-66-53
18-day
(non-inoc.)
Redwing
18-day
(inoc.)
S-66-53
18-day
(inoc.)
Origin
−
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
+
Front

Fig. 49. Electrophoretic patterns of esterase isoenzymes of dialyzed crude extracts ('A_2') from inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53.

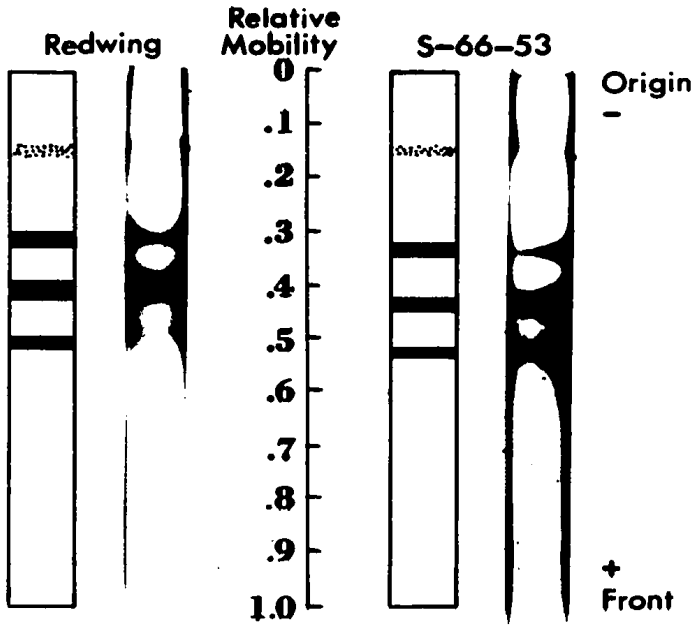
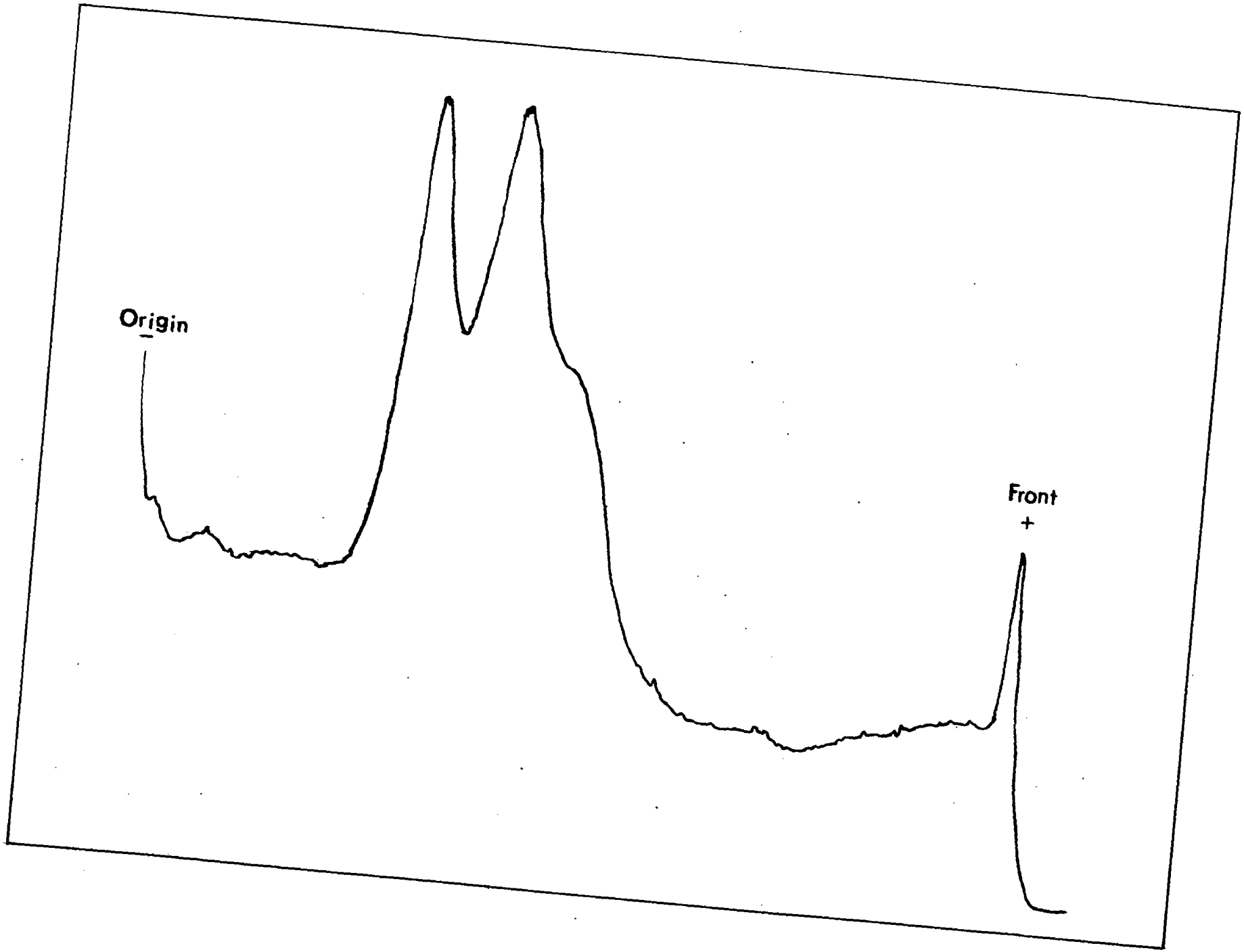

Redwing
Relative Mobility
S–66–53
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Origin
–
+
Front

Fig. 50. Typical scan for the electrophoretic pattern of esterase isoenzymes.


Origin
Front
+

wing (Fig. 46). This band however, was not observed in any of the rest of esterase isoenzymes results (Figs. 47-49). Inoculation did not seem to affect the patterns of esterase isoenzymes.

Any isoenzyme band that was observed in one variety but not in the other is marked with ◂ ▸ in the figures. Isoenzyme bands observed either in the non-inoculated or the inoculated cotyledons of each variety are marked with (*).

(5) Catalase activities: Catalase specific activities (units/mg of protein) assays were made using dialyzed crude extracts prepared from frozen and non-frozen plant materials.

The results for catalase specific activities (units/mg of protein) in 'A_2' prepared from frozen non-inoculated and inoculated cotyledons of Redwing and S-66-53 are shown in Table 9, and specific catalase activities for the non-frozen non-inoculated cotyledons of 9-day-old seedlings are shown in Table 10. Each value is the average of two assays.

Catalase specific activities of non-inoculated cotyledons of 9-day-old seedlings (Table 9) were much higher in Redwing than in S-66-53. However, activities were not detected for non-inoculated and inoculated cotyledons of 18-day-old seedlings of both varieties. In 'B' activities were not detected for non-inoculated and inoculated cotyledons of Redwing and S-66-53.

Catalase specific activity in the non-frozen non-inoculated cotyledons of 9-day-old Redwing seedlings were higher than that of S-66-53 (Table 10). The results in Tables 9 and 10 are in agreement indicating that catalase activities are much higher in Redwing (susceptible) than in S-66-53 (immune).

Results for catalase specific activities of 'A_3' from non-frozen non-inoculated cotyledons of 9-day-old seedlings of Redwing and S-66-53 and their F_1 hybrid (S-66-53 x Redwing) are shown in Table 11. Each value is the average of two assays.

Catalase specific activity was higher in Redwing than in S-66-53 and the F_1 hybrid (S-66-53 x Redwing). The F_1 hybrid had a lower catalase specific activity than each of the parent varieties.

Catalase specific activities for 'A_3' prepared from non-frozen cotyledons, the first pair of leaves above the cotyledons, and the uppermost pair of leaves of 18-day-old seedlings of Redwing and S-66-53 are shown in Table 12.

Catalase specific activities for cotyledons and the adjacent pair of leaves of 18-day-old seedlings were not detected in both varieties supporting data presented in Table 9. However, catalase activity was detected in the uppermost pair of leaves of 18-day-old seedlings. This catalase activity was higher in Redwing than in S-66-53 supporting data presented in Tables 9, 10,and 11.

Table 9

Catalase specific activity (units/mg of protein) in the dialyzed crude extracts ('A_2') and in the 50 - 90% $(NH_4)_2SO_4$ - precipitated protein fractions from frozen non-inoculated ('B') and inoculated cotyledons of Redwing and S-66-53 seedlings.

Age of Seedlings (days)	Catalase specific activity of 'A_2' units/mg of protein		Catalase specific activity of 'B' units/mg of protein	
	REDWING	S-66-53	REDWING	S-66-53
9(non-inoculated)	*A 13.20 *B 3.14	*A 2.91 *B 1.46	no activity	no activity
18(non-inoculated)	no activity	no activity	no activity	no activity
18(inoculated	no activity	no activity	no activity	no activity

Table 10

Catalase specific activities (units/mg of protein) in the dialyzed crude extracts ('A_2') prepared from non-frozen non-inoculated cotyledons of 9-day-old seedlings of Redwing and S-66-53 seedlings.

Variety	Catalase specific activity units/mg of protein
Redwing	8.61
S-66-53	1.42

* A and B are values for two different experiments.

Table 11

Catalase specific activity (units/mg of protein) in the dialyzed crude extracts ('A_3') from non-frozen cotyledons of 9-day-old seedlings for Redwing, S-66-53 and F_1 hybrid (S-66-53 x Redwing).

Variety	Catalase specific activity units/mg of protein
Redwing	3.21
S-66-53	1.90
F_1(S-66-53 x Redwing)	0.84

Table 12

Catalase specific activity (units/mg of protein) for dialyzed crude extracts ('A_3') from non-frozen cotyledon, the first pair of leaves above the cotyledon and the two uppermost leaves of 18-day-old seedlings of Redwing and S-66-53.

Plant Material	Catalase specific activity units/mg of protein	
	Redwing	S-66-53
Cotyledons	no activity	no activity
1st pair of leaves above cotyledon	no activity	no activity
uppermost pair of leaves	1.04	0.72

DISCUSSION

Resistance of plants to microorganisms is the rule rather than the exception (Yarwood 1967). This principle implies that every plant is expected to resist a great number of species and races of micro-organisms. At the same time plants of any one species may act as a host for a variety of pathogens each comprising a considerable number of strains. The first demonstration that resistance to any plant disease may be inherited was made shortly after the rediscovery of Mendel's laws of hereditary in 1900 by Biffen (1905). He studied inheritance of disease resistance to the yellow rust fungus (*Puccinia glumarum*) in different varieties of wheat. The interrelationship between hosts and their parasites have been studied extensively by Flor (1955) using flax - *Melampsora lini* system. He concluded that for each genetic locus in the host, controlling resistance and susceptibility, there is a specific and related locus in the parasite that governs its virulence and avirulence. This gene-for-gene model appears to be operating in many host-parasite interactions (Person 1967).

Previous studies on the nature of resistance in plants tend to ignore the importance of the genetic factors. Since Flor (1956) indicated the importance of using isogenic lines in studies on nature of resistance in plants to parasites, very few studies have followed this approach. In the present investigation two near-isogenic varieties, Redwing (susceptible) and S-66-53 (immune),which differ considerably in their reactions to the same race '300' of the rust fungus *M. lini*, were used. S-66-53 contains the dominant homozygous alleles *RR*, while Redwing has the recessive *rr*.

Uredospore-inoculated cotyledons of Redwing showed flecking and

prominent uredia sori. The immune variety, S-66-53 did not show any conspicuous flecking upon inoculation.

Host-parasite interface

The cytological data of the light and electron microscopy will be discussed together.

Uredospores of *M. lini* race '300' germinated equally well on each of the two varieties. This would rule out any inhibitory effect by the immune variety on uredospore germinations. Hart (1962) showed that uredospores of *M. lini* germinated readily in distilled water and the presence of host tissue (whether it is susceptible or resistant tissue was not specified) did not stimulate germinations of spores appreciably. She also observed that uredinical germ tubes entered the stoma of Argentine flax which was described as a practically immune variety.

Germination of uredospores of *Puccinia graminis tritici* was reported to occur on water solely at the expense of endogenous resources, chiefly lipids (Shu *et al.* 1954; Gottlieb and Caltrider 1962). In the present study oil globules present in uredospores and germ tubes were not observed except in some mycelial cells and haustoria developed in Redwing where they appeared as dark stained bodies. This might be an indication of lipid consumption during early stages of fungal development (Manocha and Shaw 1967). The presence of dark stained bodies in some fungal cells and haustoria might represent new synthesized lipids (Manocha and Shaw 1967). In S-66-53 oil globules in the hyphal fragments two days after inoculation could indicate a decrease in lipid consumption due to limited fungal growth. Manocha and Shaw (1967) observed that the oil globules decreased rapidly in developing intercellular mycelium of *M. lini* in a

susceptible flax variety 'Bison'. However, this assumption was not confirmed in these studies since no intercellular mycelium was observed in Redwing two days after inoculation. It has been shown (Shaw 1963; 1964; Staples and Wynn 1965; Dunkle *et al.* 1969; 1970; Trocha and Daly 1970), that germination and early stages of fungus development can be sustained by the metabolic pool within the uredospores and independent of the host.

The fungus established itself in Redwing after entering the host and produced extensive intercellular mycelium and prominent haustoria. Mycelial cells appeared to adhere to host cells with material, presumably of fungal origin, forming a cushion-like structure. Possibly host cell wall penetration and subsequent invagination of the cell is essentially enzymatical (Stavely *et al.* 1969) or enzymatical and mechanical (Edward and Allen 1970) as was described in *Erysiphe*-barley and *Erysiphe*-red clover systems respectively. In the present studies, some haustoria showed two nuclei indicating the dikaryotic nature of the fungus in this stage of the life cycle; similar results were obtained by Manocha and Shaw (1967).

Haustoria appeared to be encapsulated and, hence separated from the host cytoplasm but in close contact with it. It should be noted that haustorial structures were not observed in inoculated cotyledons of S-66-53. Ehrlich and Ehrlich (1963) suggested that at least a portion of the encapsulations in *Puccinia graminis tritici* might be fungal cytoplasm. They described "channel-like areas" in the haustorial wall and into the encapsulation connecting the haustorial proper with the encapsulation suggesting that these "channel-like areas" may permit the passage of thin strands of cytoplasm, or an exchange of metabolic products

between the host and the parasite. No evidence for such organization in *M. lini* was obtained. Vesicular structures were observed at the outer layer of encapsulation. Whether these vesicles originated from the encapsulation boundary and dispersed into host cytoplasm (Ehrlich and Ehrlich 1963), or formed in the host cytoplasm and moved to the host plasma membrane, and discharged their contents into the zone of apposition i.e. 'encapsulation' (Peyton and Bowen 1963; Berlin and Bowen 1964) was not clear. Such dynamic process can not be clearly understood in fixed killed cells. Electron microscopy in conjunction with autoradiography could possibly determine the nature and the origin of these vesicles in order to understand the nature of host-parasite interactions. While this manuscript is being prepared, a report by Ehrlich and Ehrlich (1970) applying the above suggested approach was published. They found that radioactivity was transferred from wheat rust uredospores to host chloroplasts and cell walls.

The sheath observed surrounding the haustorial neck was found to be continuous with the host cell wall, in the cells of the inoculated cotyledons of Redwing. This was in agreement with previous reports in several host-parasite interactions, *Albugo candida* - radish (Berlin and Bowen 1964), *Peronspora manshurica* - soybean (Peyton and Bowen 1963), and *Puccinia graminis* - wheat (Shaw and Manocha 1965). However, Bracker (1968) in his study with *Erysiphe graminis* - barley system, observed an electron dense layer between the collar and the host wall. In the present study the sheath was found to be surrounded with a membrane continuous with the host plasma membrane analogous to previous reports in studies of above and other host parasite systems (Stavely *et al.* 1969; Van Dyke and Hooker 1969). Electron micrographs of cross-sections in developed haustoria did not show the sheath, indicating that it did not enclose the

entire haustoria and the developing haustoria penetrated the sheath. Penetrations of the sheath by the developing haustoria is essential to establish nutritional relationship between the host and the pathogen. Peyton and Bowen (1963) observed what they have termed necrotic haustorium in which the haustorium was completely enclosed by the sheath suggesting that it could be a resistant mechanism by the host. Stavely *et al.* (1969), in their studies involving genetically related clones of red clover, did not find any walled-off haustoria of this type in either resistant or the susceptible lines infected with *Erysiphe polygoni*. Moreover, Van Dyke and Hooker (1969) in their studies on susceptible and resistant isogenic hybrids of corn, infected with *Puccinia sorghi*, concluded that physical boundaries (sheath matrix and sheath membrane) did not appear to be predominant factors in determining the compatibility or incompatibility between host and parasite.

The development of the endoplasmic reticulum close to the haustoria could be a response induced by the fungus in the susceptible host Redwing. The development of endoplasmic reticulum around haustoria has been observed in a susceptible flax variety (Bison) infected with *M. lini* (Manocha and Shaw 1967), and resistant and susceptible wheat varieties (Khapli and Little club respectively) inoculated with *Puccinia graminis* uredospores (Shaw and Manocha 1965). Shaw and Manocha (1965) suggested that such endoplasmic reticulum might be associated with the secretion of the encapsulation. However, it could be induced by haustoria, to produce special metabolites.

Haustoria appeared to contain ribosomes which indicate as suggested by Hilu (1965), that haustoria may serve as organs for synthesis as well as for absorption.

Multiple membrane structures were observed in both flax varieties as well as in the rust fungus *M. lini*. These structures were similar to the 'sac-like bodies' observed by Weintraub and Ragetli (1966) and to the multimembrane body found by Tu and Hiruki (1970). The function of such structures is not as yet known.

Nine days after inoculation with *M. lini* uredospores, uredia were formed on Redwing. Uredospores contained oil globules and two nuclei, as was found by Manocha and Shaw (1967). At this stage parasitism on Redwing appeared to enhance senescing of the host cells with the result that they became nearly devoid of cell organelles. Chloroplasts observed in such cells, contained distorted lamellar structures. Abnormal structure of chloroplasts was reported in *Glycine max* infected with *Pernospora manshurica* (Peyton and Bowen 1963). The results of Van Dyke and Hooker (1969) showed that the chloroplasts of *Zea mays* did not undergo structural changes upon infection with *Puccinia sorghi*. However, Berns and Rounds (1970) indicated that the structural changes in cell organells do not necessarily cause functional changes in such organells. They reported that structural changes in mitochondria induced by 'laser' did not alter enzyme levels in such mitochondria.

In the near-isogenic immune variety S-66-53, the limited fungal growth observed in two days after inoculations near the site of penetration consisted of few cells. These few fungal cells were larger in size than those observed in inoculated cotyledons of Redwing, indicating that fungus in the immune host was not capable of cell division or proliferation. The fungus also failed to produce any haustorial structures. Hilu (1965) found no haustoria were formed in incompatible hosts inoculated with parasites in spite of intercellular fungal growth. He also

observed premature death of haustoria in some other incompatible nearly-isogenic lines of corn infected with *Puccinia sorghi*. Other workers (Stavely *et al.* 1969) found that in resistant cells of red clover to *Erysyphe polygoni*, haustoria were not as well branched, contained fewer mitochondria, and the endoplasmic reticulum was not as well developed as observed in the haustoria in susceptible host cells. Van Dyke and Hooker (1969) did not find haustoria in necrotic cells of the incompatible resistant variety even though these cells were usually adjacent to haustoria-invaded cells.

The presence of numerous ribosomes in the hyphal fragments observed in the immune S-66-53 indicated that the fungus may possess the capacity of synthesizing ribosomal RNA independent of the host. Whether these ribosomes were involved in protein synthesis or not, is in question.

In S-66-53, uredospore germination and penetrations of the host appeared normal. However, the fungus failed to proliferate and to develop haustorial structures and therefore a successful nutritional relationship was not established. These undesirable changes in the fungus were probably due to an inhibitory factor(s) which may be present in the host cells prior to inoculation or it is rapidly induced after the intercellular mycelium is formed.

Also, in this immune variety few cells adjacent to the site of infection were necrotic indicating that a hypersensitive reaction had taken place. Hypersensitivity has recently been shown to occur in many imcompatible host-parasite systems (Hilu 1965; Stavely *et al.* 1969; Van Dyke and Hooker 1969). It was observed that chloroplasts in these necrotic cells of S-66-53 were abnormal, and their structures were differ-

ent from those observed in cells of the inoculated Redwing. Also, chloroplasts contained several starch grains enclosed in the lamellar structures. Stavely *et al.* (1969) found that in collapsed resistant cells, starch grains had been released from the chloroplasts into the mass of degenerated protoplasm. However, Ehrlich and Ehrlich (1962) found that the chloroplasts of susceptible and resistant varieties of wheat infected with *Puccinia graminis* degenerated similarly.

The hypersensitive reactions in S-66-53 cells were probably induced by the fungus. Therefore, the resistance mechanism in S-66-53 appears to involve (a) an inhibition, exerted by the host cells to prevent the fungus from normal development and haustoria formations, and (b) a response in the host cells to a fungus inducer(s) which result in collapse of host cells. These degenerative changes in host cells prevented the parasite establishing any successful nutritional relationship in S-66-53.

The question arises as to why the rust fungus was not capable of growing on necrotic host cells since it has been shown that *M. lini* could be grown saprophytically (Turel 1969, a and b); the artificial culture naturally, did not contain the inhibitory substance(s) (the nature of this substance(s) is not known) exerted by the resistant cells. Also, the possibility exists that it could be a lack of specific nutrient(s) and/or inhibitory substance effect(s).

From the evidence of host-parasite interaction the above mechanism of resistance in S-66-53 to *M. lini* appeared to be closely associated making this variety a highly incompatible host. These mechanisms are directly or indirectly related to the genetic expression of the R. locus.

Immunochemical comparison of antigens of Redwing and S-66-53

Antigens used in the above studies were considered to be proteins, because they were non-dialyzable, precipitated with ammonium sulfate, reacted with Folin-phenol reagent, and exhibited proteins, peroxidase, polyphenol oxidase(s), and esterase(s) isoenzyme bands in the disc gel electrophoretic studies.

Antigen-antibody studies showed four antigenic components in both varieties with no intercrossing in the zone of overlap between the precipitin lines. This indicated that the observed antigenic components were immunologically similar (Ouchterlony 1969). The minor changes in relative positions of precipitin lines may be due to differences in concentration among homologous antigens. The rate of antigen diffusion is known to be affected by the concentrations of antigens (Darcy 1965; Ouchterlony 1969; Simon 1969). Although the electrophoretic results of the antigen solutions of each flax variety did not show qualitative differences, more electrophoretic bands were obtained by this procedure than in the antigen-antibody test. The immunochemical absorption studies confirmed the similarity of the antigenic components of Redwing and S-66-53. Therefore, it appeared from the immunochemical data that the immediate product of R-gene expression cannot be detected by the above technique (assuming it is a protein). However, this does not rule out that the product of R-gene was either present in very small amounts undetectable by these tests,and/or the products of the *R*-allele (in S-66-53) and *r*-allele (in Redwing) were similar (cross-reactive), in spite of their obvious difference in function(s) (resistance versus susceptibility). The similarity (cross-reactivity) have been reported between antigens in which limited number of amino acids were substituted (Suskind and Yanofsky

1960; Yanofsky *et al.* 1961; Wagner and Mitchell 1965; Hunter 1967; Gilchrist *et al.* 1970).

Protein and enzyme studies

Total protein concentrations in Redwing and S-66-53 cotyledons decreased with age. This was in agreement with previous studies on changes in protein with age of cotyledons and leaves of many plants (Dorner *et al.* 1957; Koller *et al.* 1962; Doby 1965; Beevers and Guernsey 1966). Such a decrease in protein concentration with age of tissues could be due to proteolytic activity during autolysis (Beevers and Guernsey 1966). The resulting amino acids are translocated (Beevers and Guernsey 1966) and in part are probably recycled into protein synthesis in the same cotyledons, even during periods of net protein depletion (Young and Varner 1959). The increase of amino acid concentration in the ageing of cotyledons (Koller *et al.* 1962; Beevers and Guernsey 1966) could explain the differences observed in total protein concentrations, as determined by the Lowry *et al.* (1951) technique, even between dialyzed and non-dialyzed crude extracts. Higher protein values could be obtained by the presence of color-contributing compounds, such as tyrosine, tryptophan, free nucleotides (Lowry *et al.* 1951) and sucrose (Gerhardt and Beevers 1968). Sekhon and Colotelo (1970) had to increase the amount of protein, as determined by Lowry method, in order to detect protein bands in disc gel electrophoresis in their studies on ageing cultures of a ltb.

The protein concentration in cotyledons of the two near-isogenic varieties were generally at the same level, for the same seedling age. The hypothesis put forward by Rubin and Artsikhovskaya (1963) that resistant varieties have much higher protein concentration (about four times)

than in the susceptible varieties was not valid in these studies. In studies reported by Rubin and Artsikhovskaya (1963), cabbage varieties, unrelated genetically, were compared. Such studies that ignore the genetic control of resistance by comparing varieties of completely different genotypes do not necessaily explain the actual resistance mechanism involved.

The protein bands of both flax varieties, as determined by acrylamide gel electrophoresis, were similar. The similarity between Redwing and S-66-53 protein gives a special advantage of studying the genetic control of resistance at the molecular level. However, the differences may not have been detected at the level of protein concentration (300 γ) applied for each gel. Similar results were obtained by Hilty and Schmitthenner (1966) who found no differences, by gel electrophoresis, in protein bands between nearly isogenic varieties of soybean. Stavely and Hanson (1967) also found no qualitative differences in gel electrophoretic protein bands in genetically related clones of red clover before or after inoculation with powdery mildew.

Inoculation with *M. lini* did not appear to affect protein quantitatively or qualitatively in the two near-isogenic varieties. The possibility exists that *M. lini* protein or any other protein(s) that may have been induced by infection, was diluted considerably and masked by host protein.

The enzymes, studied were those which are implicated in phenolic oxidation and biosynthesis (peroxidase, and polyphenol oxidase(s)), as well as enzymes that could release phenols from a larger aromatic compound (β-glucosidase and esterase). Also, catalase was studied for its possible competition with peroxidase *in vivo*.

The possible involvement of phenolic compounds in disease resistance was recognized several decades ago by Newton and Anderson (1929). Phenol oxidation products (quinones) are extremely fungitoxic (Kosuge 1969). In living tissues, preformed resistance factors (i.e. phenols) are either considerably more fungitoxic than phytotoxic, or they are bound in a non-toxic form from which a toxic moiety is released after injury or infection(Rohringer and Samborski 1967). Some resistant plants were found to have either higher concentration of certain aromatic compounds than the susceptible varieties (El Naghi and Linko 1962; El Naghi and Shaw 1966), or they actively synthesize more phenolic compounds during inoculation with the pathogen than in the susceptible plants (Rohringer and Samborski 1967; Biehn *et al.* 1968, a and b; Kosuge 1969).

Peroxidase was reported to be widely distributed in plants (Saunders *et al.* 1964; Shannon 1969), though its exact biological function is not clearly understood (Galston and Davies 1969; Scandalios 1969). Although peroxidase has been considered to have an active role in contributing to disease resistance in certain host-pathogen interactions, no biochemical role has been proposed to account for this relationship (Jennings *et al.* 1969; Seevers and Daly 1970 b). However, peroxidase was found to be involved in phenol oxidations to fungitoxic quinones (Farkas and Kiraly 1969; Rubin and Artsikhovskaya 1964; Kosuge 1969; Veech 1969), and phenolic biosynthesis (Conn 1964; Hayaishi and Nozaki 1969). Peroxidase was found to be involved in the oxidative inactivation of biologically active proteins (Sizer 1953; Chance 1954), as well as the growth hormone indoleacetic acid 'IAA' (Goldacre 1951; Hinman and Lang 1965; Yamazaki *et al.* 1968) and possibly IAA biosynthesis *in vitro* (Riddle and Mazelis 1964). Also, peroxidase was found to be involved in lignin biosynthesis

(Schubert 1965; Brown 1966; Asada and Matsumoto 1967) and as terminal oxidase (Jennings *et al.* 1969).

The level of peroxidase activity in potato tissues before infection was reported to be positively correlated with resistance to *Phytophthora infestans* Rubin *et al.* 1947; Kedar 1959; Umaerus 1959; Fehrman and Dimond 1967). Lovrekovich *et al.* (1968) found that peroxidase of tobacco tissues appeared to be involved in the primary defense mechanism against *Pseudomonas tabaci*. A similar correlation was found between the increase in peroxidase activity or peroxidase formation and disease resistance of plant tissues after infection (Uritani and Stahmann 1961; Loebenstein and Linsey 1963; Rudolph and Stahmann 1964; Tomiyama and Stahmann 1964; Weber and Stahmann 1964; Andreev and Shaw 1965; Clare *et al.* 1966; Weber and Stahmann 1966; Maxwell and Bateman 1967; Macko *et al.* 1968; Rautela and Payne 1970).

Quantitative and qualitative determinations of peroxidase were made in non-inoculated cotyledons of 9-, 15-, 18-, and 24-day-old seedlings as well as in 9-day inoculated cotyledons (i.e. 18-day-old seedlings) to test the role peroxidase might have in resistance or susceptibility in the two near-isogenic flax varieties Redwing and S-66-53.

Peroxidase activity was found to increase significantly with the age of the cotyledons in both Redwing and S-66-53. Kawashima *et al.* (1967) observed similar increase in peroxidase activity in tobacco leaves as they aged. However, increase in activity does not necessarily mean *de novo* synthesis of the enzyme. Increase in enzyme activity could be a result of activation of a previously present zymogen or due to general protein catabolism in the cell (Filner *et al.* 1969) and hence, the specific activity of the enzyme would increase. The increase of peroxidase

activity with age of the cotyledons could be related to an increase in auxin concentration. Increased peroxidase activity with age of the tissues was shown to be induced by elevated auxin levels resulting in oxidative inactivation of the auxin (Galston and Dalberg 1954; Siegel and Galston 1967; Galston and Davies 1969). Siegel and Galston (1967) indicated that within any given plant organ, the development of peroxidase activity was a specific and orderly process, increasing from the young to the old cell. The destruction of IAA by peroxidase would have the effect of repressing certain genes which would otherwise be derepressed (activated) at that stage (Pandey 1967).

The peroxidase activity was slightly higher in the crude extracts of healthy Redwing than that of S-66-53 cotyledons which might indicate that Redwing had a slightly higher concentration of auxin. This slightly higher peroxidase activity in Redwing could stimulate fungal growth once the fungus penetrated cells of this susceptible host (no haustoria were formed in S-66-53). Zafar and Colotelo (1969) found that active horseradish peroxidase accelerated mycelial growth of the fungus *Plenodomus meliloti, in vitro.*

Peroxidase was found to be present in isoenzyme form as determined by gel electrophoresis. Peroxidase isoenzymes have been reported in plants (Shannon 1969; Scandalios 1969), in fungi (Saunders *et al.* 1964; Sekhon and Colotelo 1970), and also in bacteria and animals (Saunders *et al.* 1964). The number of peroxidase isoenzyme bands did not differ between the two near-isogenic varieties in extracts of cotyledons of various ages. These results were in agreement with those reported by Stavely and Hanson (1967) in which they found no differences in peroxidase isoenzymes between resistant and susceptible clones of red clover of genetically related

background before inoculation.

Inoculations did not appear to affect peroxidase quantitatively or qualitatively in either of the two near-isogenic flax varieties. These results were not in agreement with several previous reports in other host-parasite interactions as reviewed by Lovrekovich *et al.* (1968) in which it was reported that inoculations have increased peroxidase activity and/or the number of isoenzyme bands. Also, the present data were in disagreement with Andreev and Shaw (1965). They observed that cotyledons of a resistant flax variety (Bombay inoculated with *M. lini*) developed a premature appearance of two isoenzymes that appeared in later stages in the non-inoculated cotyledons. Their data obtained from genetically different varieties and therefore, it is not comparable with data obtained in these studies. Novacky and Hampton (1968) found no new peroxidase isoenzymes were induced in some hosts by either senescence or virus infection. However, Jennings*et al.* (1969) found that increased activities of peroxidase were associated with susceptibility in maize - *Helminthosporium* interaction. Stavely and Hanson (1967) observed an increase in the number of peroxidase isoenzymes in susceptible clones of red clover but not in the resistant clones of a genetically related background. Seevers and Daly (1970 b) concluded from their studies in two near-isogenic lines of wheat that peroxidase activity induced by *Puccinia graminis* did not appear to be correlated with resistance or susceptibility as measured by infection type. They suggested that a second type of resistance mechanism, which limits pustule numbers, might involve peroxidase. However, in a later publication (Daly *et al.* 1970) they concluded that peroxidase was not involved in disease resistance.

Polyphenol oxidase(s), like peroxidase, was reported to be involved in the oxidation and biosynthesis of phenolic compounds (Conn

1964; Hayaishi and Nosaki 1969; Kosuge 1969). Also, it was implicated in lignin biosynthesis (Schubert 1965) and terminal oxidation (Hayaishi and Nozaki 1969; Jennings *et al.* 1969). Although the precise role *in vivo* systems is not known (Hayaishi and Nozaki 1969) polyphenol oxidase(s) or phenolase complex was reported in plants to catalyze the hydroxylation of monophenol to o-diphenol (cresolase activity) and the dehydrogenation of o-diphenol to o-quinone (catecholase activity) (Conn 1964). The phenolase-polyphenol system has also been studied in connection with the inactivation of extra cellular enzymes produced by pathogens (Deverall and Wood 1961; Kúc *et al.* 1967). It was observed that tissues of various plants infected with viruses, bacteria, or fungi are characterized by an increase in polyphenol oxidase(s) activity (Farkas and Király 1962; Hyodo and Uritani 1966; 1967; Lovrekovich *et al.* 1967).

The healthy cotyledons of 9-, 15-, 18-, and 24-day-old seedlings of the two near-isogenic flax varieties Redwing and S-66-53 did not show any polyphenol oxidase(s) activity when L-tyrosine was used as substrate. However, polyphenol oxidase(s) activity was observed when the activity assays were made using catechol as substrate. This might indicate that flax polyphenol oxidase(s) did not hydroxylate monophenols to diphenols, and therefore the activity would be attributed chiefly to catecholase. However, polyphenol oxidase(s) activity in Redwing and S-66-53 was low, indicating that flax could be classified on the basis described by Saunders *et al.* (1964), as plants with a powerful peroxidase but weak in polyphenol oxidase(s) systems. Although, polyphenol oxidase activity was generally low (may be due to the presence of inhibitory substances) S-66-53 contained slightly higher activity than Redwing when the crude extracts were compared but not in the partially purified protein fractions whose activity

were greatly reduced in both varieties. Polyphenol oxidase isoenzymes have been reported in plants (Shannon 1969), fungi (Jolley and Mason 1965) and animals (Holstein *et al.* 1967). Stahmann *et al.* (1966) found that extracts of sweet potato showed isoenzymes by gel electrophoresis while they were not able to assay the enzyme activity spectrophotometrically or manometrically using the same substrate, catechol. They have suggested that this effect could be due to the presence of inhibitory substances in the extracts and the inhibitory substance was removed by electrophoresis. Disc gel electrophoretic studies showed the same number and pattern of polyphenol oxidase isoenzymes in non-inoculated cotyledons of both varieties.

M. lini inoculated cotyledons of Redwing and S-66-53 did not show any increase in polyphenol oxidase(s) activity, or the number and pattern of isoenzyme bands. These results were not in agreement with reports in other host-parasite interactions. Weber *et al.* (1967) found that polyphenol oxidase activity is increased in a resistant sweet potato variety but not in a susceptible variety inoculated with *ceratocystis fimbriata*. Also, Rautela and Payne (1970) found that o-diphenol oxidase activity increased in resistant tissues of sugar beets when inoculated with *Cercospora beticola*. The number of polyphenol oxidase isoenzymes was found to increase upon infections or wounding, (Hyodo and Uritani 1967). However, Jennings *et al.* (1970), in their studies with two genetically related hybrids of corn, suggested that the increase in polyphenol oxidase activity was associated with susceptibility and not resistance to *Helminthosporium carbonum*.

It is suggested that the levels of peroxidase and polyphenol oxidase(s) activity might be involved in a general type of resistance

mechanism against non-specific microorganisms. Thus, this suggested resistance mechanism was not controlled by the R-locus. Previously reported increases in activity or number of isoenzymes bands of peroxidase or polyphenol oxidase(s) in resistant or susceptible varieties could merely be due to host cell senescence or collapse, and may represent symptoms rather than a cause of resistance or susceptibility. Senescence and virus infection was found to increase activity and the number of isoenzymes (Bates and Chant 1970). Cell collapse initiated by infection was found to increase peroxidase and polyphenol oxidase(s) activity in the lesions *per se* (Maxwell and Bateman 1967). Wounding and cell injury was found to increase activity of peroxidase (Galston and Davies 1969) and polyphenol oxidase(s) (Hyodo and Uritani 1967). However, only few cells of the immune variety S-66-53 were affected and collapsed upon inoculation. Any increase in peroxidase or polyphenol oxidase activities in such cells would not be significant in extracts of inoculated cotyledons. Further development of microtechniques to study cells directly affected by the pathogen would help in understanding nature of host-parasite interaction.

Treatment with ethylene was found to induce peroxidase and polyphenol oxidase (Filner *et al.* 1969) and thus, Daly *et al.* (1970) attempted to induce increased peroxidase production in leaves of two near-isogenic lines of wheat differing in their reaction to rust fungus *P. graminis tritici* in order to determine if such interactions would alter leaf reaction to the fungus. They observed that susceptible treated leaves remained susceptible. In contrast ethylene-treated resistant leaves became completely susceptible, despite the fact of high peroxidase activity induced by ethylene and infection. They concluded that total

peroxidase activity was not causally related to resistance to wheat rust, expressed either as infection type or as pustule density.

Phenol compounds were reported to be widely distributed in plants and second only to carbohydrates in abundance; and they are generally present as glycosides and esters (Harborne 1964; Rubin and Artsikhovskaya 1964; Towers 1964; Pridham 1965; Hayaishi and Nozaki 1969). A study that involves β-glucosidase and esterase(s) would be of significant value in understanding the role of phenolic compounds in resistance and susceptibility.

β-Glucosidase was found to be widely distributed in plants (Veibel 1950). It was implicated in phenolic compounds biosynthesis (Conn 1964) in addition to its role in releasing phenols from plant glucosides (Pridham 1963). Noveroske *et al.* (1964) found that when apple leaf tissues were infected with *Venturia inaequalis*, a host β-glucosidase hydrolyzed phloridzin and yielded phloretin. Phloretin was then oxidized to yield products that arrested the development of the invading pathogens. The first oxidation product of phloridzin was found to be 3-hydroxphlordzin which was then oxidized to its o-quinones (Raa 1968). Oxidation products because of phloridzin were toxic to *Venturia inaequalis*, suggesting that these compounds represented a defense mechanism of the host against the fungus (Raa 1968).

β-Glucosidase activity was found to increase slightly with age of the cotyledons in Redwing and S-66-53 seedlings. Also, β-glucosidase activity was not significantly different between these two near-isogenic varieties. Hildebrand *et al.* (1969) observed that differences in β-glucosidase activity among various pear varieties generally had higher β-glucosidase activity than the resistant varieties; however, arbutin

(a host glucoside) occurred in greater amounts in resistant varieties than in the susceptible ones. Thus they suggested the presence of other pathways in pear by which the formation of antibiotic substances from arbutin could be accomplished. Powell and Hildebrand (1970) found that toxic substances were formed from arbutin via at least two processes, hydrolytic and oxidative.

Inoculation did not appear to affect β-glucosidase significantly in Redwing or S-66-53. Noveroske *et al.* (1964) concluded that tissue collapse must occur to permit β-glucosidase and phenol oxidase system to function and hence resistance would depend upon the rate of tissue collapse in the host-parasite interaction and not necessarily upon the amounts of glucosides and enzymes in host tissue. Since only few cells in the immune variety S-66-53 collapsed, as was shown in the host-parasite interface studies, β-glucosidase-glucosides would not be significant as a major defense mechanism in such a host.

Esterase(s) was studied for its possible role in hydrolyzing phenols bound with another moiety by an ester bond (Pridham 1963). The esterase(s) represent a group of enzymes whose substrate specificity and biological functions have not been fully defined (Fottrel 1968). Esterase(s) was reported to be present in plants in isoenzyme forms (Schwartz 1967; Fottrell 1968; Hall *et al.* 1969; Scandalios 1969; Shannon 1969). Esterase isoenzymes were reported also in fungi (Meyer and Renard 1969) and in animals (Semeonoff and Robertson 1968). Gel electrophoresis did not show significant differences between the two near-isogenic flax varieties in healthy cotyledons of various ages. The esterase(s) isoenzyme pattern did not appear to be altered with age of the cotyledons. *M. lini* inoculated cotyledons of Redwing and S-66-53 did not show changes in the isoenzyme pattern. It

appeared that esterase(s) did not have any significant role in the type of resistance controlled by the R-locus.

It appears from the above discussion that enzymes involved in oxidation of phenolic compounds (peroxidase and polyphenol oxidase(s)) and the hydrolytic enzymes which catalyzes the release of phenols from larger compounds (β-glucosidase and esterase(s) did not have a significant role in controlling rust reaction in the two near-isogenic varieties of flax. However, these enzymes might be involved in resistance to non-specific microorganisms or wounding. Also, they could control "background resistance" to *M. lini* race '300'. Even in susceptible Redwing the lesions are restricted in some manner, and therefore a biochemical element(s) of resistance could be involved. "Background resistance" could be defined as factor(s) which limit growth of the pathogens in the susceptible variety as a result of infection at one site.

Seevers and Daly (1970 a) found no significant differences in total phenolic compounds among healthy or inoculated rust-resistant or susceptible near-isogenic lines of wheat which seems to support the above suggestion. They also found from their studies and analyses of the individual aromatic compounds by paper, thin layer, or gas chromatography failed to reveal any correlation with resistance or susceptibility. Quantitative and qualitative studies of the individual phenolic compounds of these two isogenic lines would be of significance to clarify any role phenolic compounds might have in disease resistance.

The enzyme catalase was studied for its possible role in competition with peroxidase *in vivo* for the same substrate (H_2O_2) and it acts as an oxidative enzyme. Catalase is found in all plants; and any organism that employs cytochrome system, though its biological function(s)

remains unknown (Scandalios 1969). The cytochrome system derives energy from the interactions of oxygen with products in the Krebs cycle. This oxygen is reduced to hydrogen peroxide which must then be removed because of its toxic effect (Saunders *et al.* 1964). Catalase decomposes hydrogen peroxide to water and free oxygen molecules. This process was described by Chance (1951) as the "fastest biochemical event that has been yet measured directly"; one catalase molecule may decompose about 6×10^6 molecules of peroxide per second. Oxygen liberated in the tissue is utilized in terminal oxidation reactions (Doby 1965). Catalase was found to catalyze the oxidation of various hydrogen donors. (Cantarow and Schepartz 1962; Saunders *et al.* 1964). The enzyme was found to be present in isoenzyme forms in plants (Scandalios 1969; Shannon 1969), fungi (Hall 1967), and animals (Feinstein *et al.* 1968).

The importance of catalase in host-parasite interactions is not clear, however, Rudolph and Stahmann (1964) suggested that catalase of the pathogen might be a factor for virulence by depressing the activity of peroxidase involved in the defence reactions of the host. Maxwell and Bateman (1967) observed an increase in catalase activities in extracts of *Rhizoctonia* - infected bean hypocotyls and this increased activity was confined to the lesions *per se*.

Catalase was found to be much higher in non-inoculated cotyledons of 9-day-old seedlings of Redwing than in S-66-53. Therefore, it can be concluded that catalase could possibly be directly or indirectly involved in determining resistance or susceptibility of the host and directly or indirectly controlled by the *R*-locus.

Catalase activities were not detected in the partially purified protein fractions which were freeze-dried. Plant catalases have so far

defied all attempts of purification and crystalization (Scandalios 1969), and the inactivation of catalase in these experiments could have been attributed to freeze drying.

The presence of both catalase and peroxidase in extracts of cotyledons complicate understanding of their possible activities *in vivo*; this suggests that these enzymes are compartmentalized in the cell in such a manner that hydrogen peroxide required for peroxidase activity was not available for oxidation by catalase (Maxwell and Bateman 1967). The importance of compartments in regulating cell metabolism was recently discussed by Oaks and Bidwell (1970)

Non-inoculated cotyledons of the F_1 hybrid (S-66-53 x Redwing), which is heterozygous for the R-locus (Rr) and have an immune response to the rust fungus *M. lini*, were found to have lower catalase activity than cotyledons of Redwing. This would confirm the suggestion presented above in which the R-locus was directly or indirectly controlling levels of catalase activity and the reaction to the rust fungus. Extracts of non-inoculated or inoculated cotyledons of 18-day-old seedlings of Redwing and S-66-53 were found to have no detectable catalase activities. Absence of enzyme activity might be due to inactivation, inhibition, or enzyme degradation (Filner *et al.* 1969).

If catalase was actually involved directly or indirectly in controlling host reaction to rust infection, why do the resistant cotyledons stay resistant and the susceptible cotyledons remain susceptible at any age of the cotyledons in these two near-isogenic varieties regardless of the level of catalase activity. It is suggested that in the early stages of tissue development (e.g. leaves) the type of reaction (resistant or susceptible) to the rust fungus is programmed according to the genotype

of the plant. Thus catalase activity, which would be only critical during the early developmental stages of the tissue, should decrease in a sequential manner from the young to the old tissues. The results of the experiments in which cotyledons, 1st pair of leaves (youngest) of 18-day-old seedlings of Redwing and S-66-53 were assayed for catalase activity confirms the above suggestion. Catalase activity was only observed in the youngest leaves of both varieties; however, activity was highest, as expected, in Redwing which is the susceptible variety. Therefore, it was concluded that catalase was involved in determining susceptibility in such a manner that it was directly or indirectly (e.g. induced by the high H_2O_2 concentration as a result of activity of another oxidase enzyme), involved in the biosynthesis of a 'factor(s)' necessary for the normal growth of the fungus in host tissues during high catalase activity. This synthesized 'factor(s)' remained in the cell regardless of the level of catalase activity. In S-66-53 the level of catalase activity might not allow for the biosynthesis of this 'factor(s)' to take place, and therefore, the fungus would not be able to grow normally in the immune host.

The difference in catalase activity between Redwing and S-66-53 could be due to absence of certain isoenzyme(s) in S-66-53. Preliminary experiments for catalase isoenzyme detection, in acrylamide disc gel electrophoresis, using the method described by Thorup *et al.* (1961) were carried out; however catalase isoenzymes were not detected due to staining difficulties and the hampering effect exerted by evolution of free oxygen. These results were not reported in this thesis. Starch gel electrophoresis (Smithies 1959) or a modified acrylamide disc gel electrophoresis which would overcome these problems should be applied.

It appeared that resistance and susceptibility is a developmental problem and its understanding would be correlated with our knowledge of the control mechanisms and differentiations in higher plants. Genetic control of cell functions is considerably more complicated in higher organisms than in microorganisms (Britten and Davidson 1969; Filner *et al.* 1969; Wyngaarden 1970).

A hypothesis can be suggested to explain the control of resistance and susceptibility in the two near-isogenic varieties of flax in view of the data presented above. In this hypothesis the *R*-locus has a pleiotropic effect. It controls (1) preformed resistance mechanism(s) (i.e. present in the tissues before inoculation) including the level of catalase during the period in which catalase is synthesized (or activated), and (2) the induction of a hypersensitive reaction as a result of inoculation. Such a control could be accomplished if the *R*-locus produces a single cytoplasmic factor that binds two different operators each of which controls one of the above functions.

The level of catalase during its active synthesis (or activation) might be a result of a balance between the rate of synthesis (activation) and the rate of degradation (inhibition). This balance would be directly or indirectly under the control of the *R*-locus. Mutants affecting the balance between catalase synthesis and degradation has been reported in mice (Ganschow and Schimke 1970). In S-66-53 (RR), the balance between synthesis and degradation (or activation and inhibition respectively) would be at levels lower than Redwing. Since such regulation is achieved *via* a cytoplasmic product, as suggested above, the F_1 hybrid (Rr) which is immune to rust fungus similar to S-66-53, has a lower catalase activity. In older tissues catalase synthesis (or activation) is turned off, or degradation is more predominant than synthesis.

An induction hypothesis to explain the control of the hypersensitive reaction as a result of inoculation with *M. lini* is a modification of the hypothesis described by Hadwiger and Schwochau (1969). In the present hypothesis *R* (as in S-66-53) produces a repressor which is bound to the second suggested operator. This operator would control degenerative processes in host cells when derepressed by a fungus inducer upon inoculation. This inducer eventually penetrates host cell walls. In Redwing (*rr*) the repressor is altered such that it can not bind the fungal inducer and therefore, no rapid collapse of the host cells would occur. This would permit the fungus establishing a successful nutritional relationship in contrast to S-66-53. In the F_1 hybrid (*Rr*) (immune) there would be a competition between the repressor (produced by *R*-allele) and the altered repressor (produced by *r*-allele) in binding to the operator. Inoculation then as in S-66-53 would derepress the operator and induce degeneration and collapse to the adjacent host cells. Further research would be needed in order to confirm such hypothesis.

SUMMARY

Two near-isogenic varieties of flax, differing in a monogenetic factor that controls their reactions to rust fungus *Melampsora lini* (Pers.) Lev. f. sp. *liniperda* Koern race '300' were studied. The salient features resulting from this study include:

1. Cytological studies of host-parasite interface indicated that *M. lini* uredospores germinated similarily on cotyledons of the two near-isogenic varieties, Redwing and S-66-53. In Redwing the fungus formed extensive intercellular mycelium and haustoria and later uredospores. However, in S-66-53, very limited fungal growth was observed but no haustoria were formed. Inoculation of S-66-53 resulted in a hypersensitive reaction.

2. Immunological studies did not show a difference between antigens of these two near-isogenic varieties.

3. Total protein was found to decrease with age of cotyledons in Redwing and S-66-53, but no qualitative (as determined by disc gel electrophoresis), or quantitative differences were found between these two near-isogenic varieties before or after inoculation. Inoculations did not appear to change protein concentrations or the number of protein bands.

4. Phenol oxidizing enzymes; i.e. peroxidase and polyphenol oxidase did not appear to be involved in resistance or susceptibility of the type controlled by the R-locus. Also enzymes that release phenolic moiety from larger compounds, i.e. β-glucosidase and esterase(s) did not appear to be related to resistance or susceptibility in these near-isogenic varieties.

5. Catalase activity was found to be much higher in Redwing than in S-66-53 or in an F_1 hybrid (immune) of these varieties. Older tissues, whether inoculated or not, did not show any detectable catalase activity. Younger tissues contained higher catalase activity than the older tissues. It was suggested that catalase might be directly or indirectly related to the control of Redwing susceptibility by involving in the biosynthesis of a 'factor(s)' necessary for normal growth of the fungus.

6. A hypothesis was proposed to explain the preformed resistance mechanism(s) including catalase, and the induction of the hypersensitive reaction.

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