HOST:PARASITE INTERACTIONS OF RUSTS AND AXENIC CULTURE OF *MELAMPSORA* SPECIES

by

WILLIAM DAVID LANE

B.Sc.(Agr.), University of British Columbia
M.Sc., University of British Columbia

A Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in the
Department of Plant Science

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December, 1974

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Plant Science
University of British Columbia
Vancouver, British Columbia V6T 1W5

Date Jan 1, 1975

ABSTRACT

The thesis is composed of two sections. The first section comprising Chapters I and II is designed to examine possible interactions between isolated flax protoplasts and germinating rust urediospores or axenically grown rust mycelium in an attempt to obtain evidence for or against the theory that toxins play a role in resistance and susceptibility in the flax:flax rust system. A number of previously published observations form the basis of the toxin theory of resistance. These can be summarized as follows: necrotic cells are found surrounding the flax rust infections; the cytoplasm of the rust and the host never come into direct contact; plant cells some distance removed from the site of infection become necrotic; the necrotic area around an infection can be extended away from the site of infection when an electric current is passed through the leaf, the necrosis extending in the direction of the positive pole; and the characteristics of the necrotic area vary depending on the virulence and resistance genes involved in the interaction.

Chapter I describes the isolation of flax protoplasts and their growth and development in vitro. The medium used and other cultural conditions were found to influence both the type of development and the longevity of cultured protoplasts. When cultural conditions were optimal, some protoplasts divided mitotically, and many remained alive for a

month or longer. Information obtained from these experiments formed the basis of the experimental procedures employed in the work described in Chapter II.

In Chapter II the toxin theory of resistance and susceptibility was examined using a protoplast bursting bioassay to detect any fungal toxins, if they were present in the test solution. Protoplasts are suitable for a bioassay of this kind because they are fragile and sensitive and burst when exposed to toxic substances. The bursting response is easy to detect and to quantify. Because axenic cultures of flax rust are now available a search for evidence of extracellular products with toxic properties is therefore possible. Spore germination medium is also a possible source of fungal toxins. If the protoplasts isolated from either resistant or susceptible varieties, or both, were burst when incubated with exudate from axenic cultures or spore germination medium this would constitute evidence in support of the toxin theory.

The search for toxic substances in both axenic culture exudate and spore germination medium yielded negative results. These, together with other previously published results, argue against the involvement of toxins in the flax: flax rust system. An alternative explanation, consistent with the observations which originally suggested the toxin theory, is that, when the fungus invades the intact leaf, toxins are produced by the host plant. This proposal is discussed.

The second section of the thesis, comprising Chapters III and IV, describes techniques for growing rusts axenically. In Chapter III a technique is described for the isolation of colonies of flax rust from infected cotyledons. The technique depends on the digestion of host cell walls with hydrolytic enzymes and washing the liberated colonies free from adhering flax protoplasts. Using this method it is possible to collect large numbers of flax rust colonies with only a few host cells adhering to them. The isolated colonies can be used as a source of uncontaminated fungal tissue. Axenic cultures were established using colonies isolated in this way as inoculum.

The results in Chapter IV are an extension and modification of those in Chapter III and describe the axenic culture of poplar rust for the first time. Surface sterilized leaf pieces of black cottonwood leaves centered on uredial infections of poplar rust (Melampsora occidentalis) were placed pustule side up on a completely defined agar base medium. All the pustules grew for the first two weeks and after 4 months about 30% of them became established as vegetative axenic culture colonies on the agar medium. The leaf tissue did not grow. The axenic cultures were successfully subcultured to fresh medium. Some produced spores in culture. The colonies were capable of reinfecting excised leaves of the host in vitro. The new axenic culture techniques described in Chapter III and IV are simpler than previous methods and have several other advantages.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

As I near the end of my doctoral studies I feel particularly grateful and fortunate to have had Dr. Michael Shaw as my supervisor. He has provided encouragement and help and his example has been an inspiration. I would also like to thank the members of my committee for prudent advice. They were: R.J. Copeman, G.W. Eaton, P.A. Jolliffe, V.C. Runeckles, G.H.N. Towers and P.M. Townsley. Gayle Smith typed the thesis and helped in other ways; I thank her.

I was supported for the last three years with a Leonard S. Klinck Fellowship awarded by the University of British Columbia. Chapters III and IV have been previously published in the Canadian Journal of Botany.

GENERAL INTRODUCTION

The rust diseases are perhaps the most destructive and important diseases of man's crop plants. Two important characteristics of pathogenesis by rust fungi, which contribute to making rust diseases so important, are the specificity with which races of rusts attack varieties of crop plants and the highly evolved obligately pathogenic nature of the rust fungi. Rust researchers have made efforts to understand the specificity of rust by attempting to explain the mechanism of resistance and susceptibility of host plants to rusts. The question of the obligately pathogenic nature of rusts has been approached by culturing rusts axenically apart from cells of the host plant which the rust normally infects.

Historical Aspects of Rust

Chester (8) began his book by summarizing early accounts of rust; several of these are described here.

The origin of the rust fungus was documented in Roman folklore by Ovid (43 BC - 17 AD). According to the story a poor farm couple in the Latin state of Carseoli had a willful son who caught a fox plundering his chicken yard. The boy wrapped the fox with straw and hay, set this on fire, and turned the fox loose. As punishment for this sin, the gods visited mankind with rust. The Romans prayed and made sacrifices to two rust gods, Robigus and Robigo. Rust and

rust epidemics are also mentioned in many places in the Bible. In more modern times Shakespeare refers to it in "King Lear" and as science and botany were developed many people studied and wrote about it. Chester (8) quotes Fontana (1767) as follows:

> "If the stalk and leaves are attacked by this terrible malady, the best set (of kernels), promising a heavy yield, is reduced to nothing or almost nothing because such great numbers of the greedy and gluttonous plants (rust) absorb nearly all the nutritive humor of the grain, causing it to become wasted and consumed because of the loss of the nourishing chyle."

The historical awareness of and concern with rust testify to the destructiveness and importance of this phytopathogen. Time has not lessened the dubious honor long held by rust of being the most destructive pathogen of man's food plants. Recent epidemics of stem rust occurred in 1954 in North America and in 1964 in Australia. Severe outbreaks of leaf rust are still common in the winter wheat growing areas of the United States and other countries. At the present time coffee rust is epidemic in Latin America causing the destruction of many plantations.

Most of the useful scientific research on rusts has been carried out in the last seventy-five years. Early investigations described important aspects of rust biology, the understanding of which are of paramount importance in understanding the host:parasite relations of the rusts. The best example of useful results accruing from studies

of host:parasite relations was the recognition of genes for rust resistance in crop plants. Plant breeders have effectively used this information to breed resistant varieties. This has resulted in reduction of crop losses due to rust. Additional information will contribute to reducing losses still further.

There are two outstanding objectives of physiological studies on rust pathogenesis of host plants. The first is the elucidation of the biochemical basis of the genetic specificity of resistance and susceptibility. The second is to determine the nutritional requirements for rust growth.

Satisfactory answers to both of these questions are important to the understanding of two factors which are particularly relevant to the destructiveness of rust. These are the extreme adaptability of rusts and their ability to parasitize and destroy large acreages of crops by spreading in an explosive manner when the climatic conditions are favourable. Measures used to control and prevent the destruction by rusts in the future will be based on the comprehension of these factors.

Genetic specificity of rust parasitism has been shown in a number of cases to be extreme. Often one gene in the fungus determines avirulence or virulence and the corresponding gene in the host determines resistance and susceptibility. The interaction of these genetic factors determines whether successful establishment of the rust

and pathogenesis of the host occurs or whether the interaction results in a resistant reaction. Although, little is known at the present time about the biochemical basis of these genetic interactions, the knowledge of it in genetic terms has been instrumental in the development of rust resistant varieties of a number of crops and in the prediction of which varieties are potentially vulnerable to new races of rust.

Susceptibility and Resistance

A number of hypotheses have been advanced to explain the mechanism of plant resistance and susceptibility to pathogens. These include the nutritional and the phytoalexin theories and the hypersensitivity defence reaction. Two others, which are different explanations of the specific gene product theory, have also been proposed. They are Stahmann's hybrid enzyme theory (37) and Albersheim's theory (1) that cell wall digesting enzymes produced by the fungus are regulated by the specific composition and structure of carbohydrate polymers making up the host cell wall. Others (35) have suggested that the "specific gene product" may be RNA or DNA which could be exchanged in one or both directions.

The basis of the nutritional theory of susceptibility and resistance is derived from the basic fact that the parasitized leaf is the substrate upon which the fungus depends for nutrition. It is a simple step to suggest that a susceptible host:parasite combination can establish a suitable

metabolic and nutritional environment while the resistant combination can not. The ability to grow some rusts and other classically obligate parasites in axenic culture has been argued by some as evidence against this theory. The fact still remains that the interactions of a fungus with its host may regulate fungal metabolism and nutrition in such a way as to be instrumental in determining susceptibility and resistance.

The phytoalexin theory of resistance and susceptibility became of considerable interest soon after the discovery of phytoalexins (13). These compounds (usually phenolics) are inhibitory to fungus growth and are often found in increased concentrations shortly after fungal invasion. They are always found in association with necrosis. The phytoalexin theory proposes that during resistant interactions these compounds inhibit fungal growth eventually resulting in fungal death. In the susceptible interaction they are degraded into harmless derivatives. The most important observation arguing against a primary role of phytoalexins in resistance is that their production is non-specific.

The importance of hypersensitivity in disease resistance and susceptibility has been debated since Stakman first described it in 1915. Although hypersensitivity is an ill-defined term, it is generally considered to be the rapid necrosis of a limited number of cells around the site of infection. The theory proposes that rapid host cell death

isolates the invading pathogen and causes its death. Several observations have lessened the significance of this theory. First, hypersensitivity is not always associated with resistance, and second, incipient infections surrounded by necrosis for as long as twenty days are known to still be alive. Kiraly (26) in a recent article has argued against this theory. When it was discovered that there was a complementarity of genes in the fungus and host which determined resistance and susceptibility, one gene determining virulence in the pathogen and one gene determining resistance in the corresponding host (20), theories proposing single gene products of both the fungus and its host become important. Stahmann (37) suggested that complementary genes in the host and pathogen carry information determining subunit structure of the same enzyme in both the host and the pathogen and that these play a primary role in regulating metabolism during host:parasite interaction. Exchange of subunits in one or both directions would make possible a hybrid enzyme with properties different from the corresponding enzyme in either the host or parasite. Such interactions could occur for a number of enzymes at least as great as the number of major genes controlling virulence and resistance.

During the susceptible interaction all the hybrid enzymes would contribute to the regulation of host metabolism in a manner advantageous to the fungus. In the incompatible reaction one or more of the hybrid enzymes would cause

conditions unsuitable for fungal growth and pathogenesis, thus resulting in fungal death and hence resistance.

Similar theories have been proposed in which it is postulated that DNA or RNA may be transferred from the fungus to the host or vice versa (31, 35).

Albersheim (1) has proposed a theory which involves host cell wall specificity. He proposes that the composition and structure of the host cell wall carbohydrates determine the pathogen's ability to produce enzymes capable of degrading it. He discusses evidence demonstrating the extreme specificity of carbohydrate polymers as antigens as well as cell wall polymers as substrates for degradative enzymes. The regulation of the degradative enzymes is also very specific and specific products of cell wall degradation such as disaccharides could easily be specific effectors and repressors of the fungal degradative enzymes. Albersheim's theory satisfies the theoretical requirements for specific gene products from both the fungus and the host, the degradative enzymes of the pathogen and the composition or structure of the plant cell wall being the components conferring the specificity.

In Chapter II experiments undertaken to investigate the fungal toxin mechanism of susceptibility and resistance are presented. Scott (32) described the appeal of the fungal toxin theory succinctly:

> "...it is difficult to escape the conclusion that compounds are released by the invading pathogen during the early stages of infection, especially

> since it is well established that fungal cytoplasm does not come into direct contact with host cytoplasm. Such compounds would have to be readily diffusible as cells removed from the invading pathogen are affected. Yet these compounds would have to be large enough to carry specificity... Now that some rust fungi have been grown axenically, it should be possible to search their culture media for compounds which will react specifically with either a susceptible or resistant host."

As no serious attempts have been made to verify or reject this theory with respect to the rust fungi I undertook to investigate this problem.

Chapter I describes the isolation of flax protoplasts from cotyledons and their longevity and development in culture. The information and experience obtained from these studies was used in planning the experiments carried out in Chapter II.

Experiments utilizing protoplasts to study the toxin theory of resistance and susceptibility are described in Chapter II. When testing theories designed to explain the biochemical basis of the genetic specificity of resistance and susceptibility technical difficulties are encountered. These arise because, at the time when primary events determining susceptibility and resistance occur, the mass of fungal tissue is small compared to the mass of the host. Protoplasts were used, in an <u>in vitro</u> system, so that a large population of protoplasts would interact simultaneously with the rust fungus, thus permitting examination of the initial responses of the protoplasts to the rust.

Axenic Culture

Scott (32), Scott and Maclean (33) and Yarwood (45) have reviewed axenic culture of rusts and other obligate phytopathogens. Much of the information in the first part of this introduction was drawn from these sources.

Rust fungi are among the most advanced fungal pathogens. They are obligate parasites and in nature only grow on living host plants although recently several species have been successfully grown in culture. Most species of rust are only able to parasitize one, or at most, a few closely related plant species. They seldom kill infected plants outright. Rusts are able to obtain nutrients from the host and produce large numbers of urediospores in a cyclic manner with a period of only about ten days. This makes available a large inoculum pool and is instrumental in the rapid spread and the destructiveness of rust epidemics.

Strictly obligate phytopathogenic fungi are those species which grow and reproduce in nature only in association with living host plants and cannot be cultured axenically (32). Yarwood (45) calculated that fully one quarter of the known plant pathogens are obligate parasites and that over 5000 species of fungi are obligately phytopathogenic out of a total of approximately 37,000 known species of fungi. In the Phycomycetes 140 obligate pathogenic species are found in the families Peronosporaceae (downy mildews) and Albuginaceae (white rusts). Ascomycetes of the Order

Erysiphales (powdery mildews), consisting of about 90 species, are also obligate parasites. The largest number is in the Order Uredinales (rusts) which, classically, was considered to be composed entirely of obligates. This Order in the Class Basidiomycetes includes 4500 known species.

Because few obligately phytopathogenic fungi can be grown axenically (growth of organisms of a single species in the absence of living organisms or living cells of any other species (18)), difficulties are encountered in studying host: parasite interactions and mechanisms of resistance and susceptibility. If obligate fungi could be studied other than in the diseased plant, different and potentially useful approaches could be taken.

Three approaches have been considered to overcome this problem; first, studying germinating spores; second, inducing the fungus to grow out of the diseased plant or isolating it directly; third, development of axenic culture techniques using either infected host callus cultures or urediospores as inoculum.

The physiology of urediospores has been reviewed (2, 3, 9, 10, 28, 34, 36) and will not be considered in detail here. Urediospores, and to a limited extent other spore forms (32), have been used in studies of the development of the fungus and as a source of fungal tissue separated from the host, for metabolic and other studies (34).

Turel and Ledingham (39) developed a method for inducing aerial mycelium to grow from infected leaves maintained in culture. Excised aerial mycelium was used as a source of fungal tissue in metabolic studies (29, 40, 43). In this work difficulties in interpretation of results arose because of the unknown effects of excision on fungal metabolism (43).

Another technique for obtaining fungal tissue separate from that of the plant was used by Dekhuijzen et al. (18, 19). Infected leaves were ground, filtered to remove leaf fragments and the hyphae segments concentrated by settling of the filtrate in sucrose gradients. A major disadvantage of this method is the loss of cell contents from the broken hyphae and the alteration of metabolism caused by grinding.

Chapter III describes a method for the isolation of intact fungal colonies from infected leaves. This source of fungal material has distinct advantages when compared to germinated spores, aerial mycelium or fragments isolated by grinding. The technique takes advantage of a property of the rust not previously exploited, the different composition of the rust cell wall compared to that of the host. Enzymes were used which selectively digest the host cell walls; the resulting protoplasts either burst or float away from the fungal colonies leaving them intact. The technique is gentle and few host cells remain as contaminants. It is possible to obtain sufficient fungal material for many experiments,

easily and in a short time (2 gms fresh weight of flax rust fungus requires 1.5 h work).

Perhaps the greatest advantage is that greater than 95% of the colonies are viable after isolation and washing. This was demonstrated by using them as inoculum for establishing axenic cultures. The disadvantage of the method is that the possibility of altered metabolism due to the isolation remains.

Twenty-seven years ago Hotson and Cutter (23) reported the first growth in axenic culture of a rust. This breakthrough made axenic cultures a potential source of uncontaminated rust tissue. Development of techniques subsequent to Hotson and Cutter's work has made this potential a reality for a small number of rust species. In comparison with the sources of rust material described above axenic cultures have several advantages. Greater amounts of undisturbed tissue are available. Also, studies of development past the germling stage are possible.

Yarwood (45) in 1956 summed up the "state of the art" saying, "The Peronosporaceae, the Erysiphaceae, the Uredinales, Rhizella and Polystigma have still not been cultured but some lichen fungi many of the Chytridiales and at least one species of Rhytisma have since been cultured". Other reviews on axenic culture of rusts have been written since then by Brian (6), Scott and Maclean (33), Scott (32) and Wolfe (44).

To obtain axenic cultures of rusts or any other obligate parasite at least three conditions must be met:

1, separation of the rust from bacteria and other contaminants with which they are normally in close association in nature; 2, provision of a suitable and favourable nutritional environment; 3, provision of a favourable physical environment. Several approaches have been taken to fulfill these requirements resulting in successful establishment of several rusts in axenic culture.

Morel (30) first suggested using systemically infected plant callus as inoculum to initiate rust axenic cultures. This was the technique used by Hotson and Cutter (23) to grow Gymnosporangium juniperi-virginianae. The method was tedious and the success rate low. In one series of experiments, 8,840 telial galls on juniper were collected and 13,504 callus cultures initiated from them. Of these 358 were found to be systemically infected with rust. After 4 to 9 months one of the primary cultures and 6 of the many subcultures became necrotic and rust grew out of the callus onto the medium, subsequently developing into an axenic culture (15). Hotson and Cutter used Gautheret's nutrient no. 4 plant tissue culture medium modified by the addition of 3% dextrose and 500 ppm ascorbic acid. Later using the same technique Hotson and Cutter obtained 5 cultures of the rust Uromyces aritriphylli (15, 16) and one of Puccinia malvacearum (reported in a posthumous paper examined by Scott and Maclean). Although others have not been able to repeat Cutter's work, it is respected, and these three rusts are considered to be

the first which were cultured axenically.

Cronartium fusiforme has also been grown using the infected host tissue culture method (22). To initiate infected plant tissue cultures 200 gall segments were used. Mycelium grew out of one of these and the rust was subsequently established in axenic culture. A total of 24 weeks was required from culturing the galls until establishment of the axenic cultures. Aeciospores were produced in culture. Harvey and Grasham (21) have grown Cronartium ribicola in axenic culture using the infected tissue culture method. This rust was grown both on a defined medium containing glutathione and cysteine and a medium containing yeast extract, peptone and bovine albumin. Spore-like bodies formed regularly in some cultures and pathogenicity of the cultures was demonstrated.

The approach, other than use of infected tissue cultures, has been to use urediospores as inoculum. The first success with this method was reported in 1966 by Williams et al. (41) with Puccinia graminis tritici race ANZ 126-6,7, an Australian isolate of stem rust of wheat. In this paper, no spores were reported to be produced in culture. Axenic growth was confined to colonies a maximum of 1 mm in diameter and no claims were made of being able to subculture the rust by serial transfer. Later (42) pathogenicity of the cultures was demonstrated and spores were produced in culture. The first mention of successful serial subculture of Australian stem rust cultures was in 1969 (33). The medium upon which

this rust grew contained Czapek's minerals, sucrose, 0.1% yeast extract and 0.1% Evans' peptone. Subsequent work by Bushnell (7) confirmed the success of Williams et al. (41, 42).

In a comparative study of cereal rusts involving Puccinia graminis tritici, P. graminis avenae, P. graminis secalis, P. sorghi, P. coronata avenae and P. recondita triticia, Kuhl et al. (27) found that only P. graminis avenae grew, apart from the Australian stem rust isolate previously cultured. Growth was sporadic and variable but some spores were produced after several weeks. No attempts were made to serially subculture Puccinia graminis avenae.

Soon after Williams first report, Turel (38) successfully grew Melampsora lini (flax rust) in axenic culture from urediospores. Mycelial growth developed in 20 to 30% of the culture tubes which were seeded. Several cultures were reported to have survived successive transfers over a period of 5 months. Although spores were produced in culture, many of them were irregularly shaped. The medium used contained Knop's minerals, Berthelot's trace elements, sucrose, and 0.1% yeast extract. Turel's work was confirmed by Coffey, Bose and Shaw (11).

Uromyces dianthi (carnation rust) was cultured by Jones (24) from urediospores. About 65% of the inoculations developed into mycelial growths. At first growth stopped after

2-3 months, but later this did not occur. To date, pathogenicity of axenic cultures of this rust has not been demonstrated nor were spores produced in culture. The medium used contained Czapek Dox Broth, 0.2% peptone and 0.2% yeast extract. Serial subcultures of Uromyces dianthi were used subsequently in a nutritional study (24).

Coffey and Allen (12) used urediospores to initiate axenic cultures of Puccinia helianthi (snap dragon rust). When conditions were optimal, axenic cultures developed in 80-100% of flasks. Colonies reached a diameter of 12 mm after 25 weeks and could be serially subcultured after 7 to 14 weeks. This operation was more successfully done as the colonies became older, presumably because they became adapted to growth in vitro. Pathogenicity of these cultures has not been demonstrated but some thick walled teliospores were produced in vitro. Growth occurred in a medium containing the same minerals used successfully for flax rust (36), plus sucrose, defatted bovine serum albumin and either Evans' peptone, tryptone or casamino acids. The requirements for bovine serum albumin (1.5 - 2.0%) was absolute but two amino acids, glutamic acid, aspartic acid or alanine in combination with cysteine chould be substituted for the peptone, tryptone or casamino acids.

Two recent advances of considerable importance to the field of axenic culture are the development of two defined

media, one suitable for the growth of flax rust and the other for wheat rust (4), and the ability to induce sporulation of flax rust (race 3) by including amino acids (aspartic or glutamic acid) plus calcium in the medium (5). Because urediospores and teliospores are both produced in culture, the technique is particularly valuable for studying rust genetics.

Section II of this thesis describes two new approaches for the initiation of axenic cultures which avoid some of the difficulties and limitations encountered when using infected host tissue cultures or uncontaminated urediospores as inoculum. Kuhl et al. (27) discuss the difficulties of inducing urediospores to grow past the sporeling stage and become vegetative colonies. Concerning this they wrote:

> "...between germ tube elongation and the formation of saprophytic hyphae a change occurs in the expression of the rust genome with consequent reorientation of the rust metabolism. Of sporelings that do initiate saprophytic growth only a small number survive to form vigorous macroscopically visible colonies. This could be due to the extent to which developing hyphae adapt their metabolism to the needs imposed by the artificial medium. We take the view that erratic growth encountered with present methods is partly due to the lack of control over the transition process. Thus a random event determines whether the transition does or does not take place".

By obtaining mycelial inoculum from naturally infected leaves, the transition from sporeling to vegetative growth could be by-passed. As a result, variability would be decreased and cultures established more easily and it would perhaps be possible to grow species of rusts which have not been

successfully grown before.

The initial objective of the work described in Chapter III was to devise a method for separating the rust fungus tissue from the infected leaf. As well as being a source of uncontaminated fungal tissue, colonies isolated from cotyledons by selective enzymatic hydrolysis of host cell walls were used as inoculum for establishing axenic cultures of the flax rust fungus.

Chapter IV describes a method of establishing axenic cultures which has the advantages of, and is based on, the method described in Chapter III. It does not, however, require the use of enzymatic digestion. This method was used to establish axenic cultures of poplar rust for the first time.

REFERENCES

1. Albersheim, P., T.M. Jones and P.D. English. 1969. Biochemistry of the cell wall in relation to infection processes. Ann. Rev. Phytopathology 7: 171-194.

2. Allen, P.J. 1965. Metabolic aspects of spore germination in fungi. Ann. Rev. Phytopathology 3: 313-342.

3. Arthur, J.C. 1929. The plant rust (Uredinales). John Wiley and Sons, Inc., New York, 466 pp.

4. Bose, A., and M. Shaw. 1974. In vitro growth of wheat and flax rust fungi on complex and chemically defined media. Can. J. Bot. 52: 1183-1195.

5. Bose, A., and M. Shaw. 1974. Rust fungi of wheat and flax: growth on chemically defined media. Nature 251: 646-648.

6. Brian, P.W. 1968. Obligate parasitism in fungi. Proc. Royal Soc. Ser. B.: 101-118.

7. Bushnell, W.R. 1968. In vitro development of an Australian isolate of Puccinia graminis f. sp. tritici. Phytopathology 58: 526-527.

8. Chester, K.S. 1946. The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat. Chronica Botanica Company, Waltham, Mass., U.S.A.

9. Cochrane, V.M. 1958. Physiology of fungi. John Wiley and Sons, Inc., New York, 524 pp.

10. Cochrane, V.M. 1960. Spore germination in plant pathology. An Advanced Treatise II: 169-202 (J.G. Horsfall and A.E. Dimond, Eds.) Academic Press, New York, 715 pp.

11. Coffey, M.D., A. Bose and M. Shaw. 1970. In vitro culture of flax rust Melampsora lini. Can. J. Bot. 48: 773-776.

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

13. Cruickshank, I.A.M. 1963. Phytoalexins. Ann. Rev. Phytopathology 1: 351-374.

14. Cutter, V.M., Jr. 1952. Observations on the growth of Uromyces caladii in tissue cultures of Arisaema triphyllum. Phytopathology 42: 479.

15. Cutter, V.M., Jr. 1959. Studies on the isolation and growth of plant rusts in host tissue cultures and upon synthetic media. I. Gymnosporangium. Mycologia 51: 248-295.

16. Cutter, V.M., Jr. 1960. Studies on the isolation and growth of plant rusts in host tissue cultures and upon synthetic media. II. Uromyces ari-triphylli. Mycologia 52: 726-742.

17. Dougherty, E.C. 1953. Problems of nomenclature for the growth of organisms of one species with and without associated organisms of other species. Parasitology 42: 259-261.

18. Dekhuijzen, H.M., H. Singh, and R.C. Staples. 1967. Some properties of hyphae isolated from bean leaves infected with bean rust fungus. Contrib. Boyce Thompson Inst. 23: 367-372.

19. Dekhuijzen, H.M., and R.C. Staples. 1968. Mobilization factors in urediospores and bean leaves infected with bean rust fungus. Contrib. Boyce Thompson Inst. 24: 39-52.

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

21. Harvey, A.E., and J.L. Grasham. 1974. Axenic culture of the mononucleate stages of Cronartium ribicola. Phytopathology 64: 1028-1035.

22. Hollis, C.A., R.A. Schmidt, and J.W. Kimbrough. 1972. Axenic culture of Cronartium fusiforme. Phytopathology 62: 1417-1419.

23. Hotson, H.H., and V.M. Cutter, Jr. 1951. The isolation and culture of Gymnosporangium juniperi-virginianae Schw. Proc. Nat. Acad. Sci. U.S.A. 37: 400-403.

24. Jones, D.R. 1972. In vitro culture of carnation rust Uromyces dianthi. Trans. Brit. Mycol. Soc. 48: 29-36.

25. Jones, D.R. 1973. Growth and nutritional studies with axenic cultures of the carnation rust Uromyces dianthi (Pers.) Niessl. Physiol. Plant Pathology 3: 379-386.

26. Kiraly, Z., B. Barna and T. Ersek. 1972. Hypersensitivity as a consequence, not the cause of plant resistance to infection. Nature 239: 456-457.

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

28. Mains, E.B. 1917. The relation of some rusts to the physiology of their hosts. Am. J. Bot. 4: 179-220.

29. Mitchell, D., and M. Shaw. 1968. Metabolism of glucose-C^{14}, pyruvate-C^{14} and mannitol-C^{14} by Melampsora lini. II. Conversion to soluble products. Can. J. Bot. 46: 435-460.

30. Morel, G. 1948. Recherches sur la culture associee de parasites obligatoires et de tissues vegetaux. Ann. Epiphyties (II) 14: 1-112.

31. Rohringer, R., N.K. Howes, W.K. Kim and D.J. Samborski. 1974. Evidence for a gene-specific RNA determining resistance in wheat to stem rust. Nature 249: 585-588.

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

33. Scott, K.J., and D.J. Maclean. 1969. Culturing of rust fungi. Ann. Rev. Phytopathology 6: 123-146.

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

35. Shaw, M. 1967. The physiology and host parasite relations of the rusts. Ann. Rev. Phytopathology 1: 259-294.

36. Staples, R.C. and W.K. Wynn. 1965. The physiology of urediospores of the rust fungi. Bot. Rev. 31: 537-564.

37. Stahmann, M.A., W. Woodbury, L. Lovrekovich and V. Macko. 1968. The role of enzymes in the regulation of disease resistance and host-pathogen specificity. In Biochemical Regulation in Diseased Plants or Injury. pp. 264-274. T. Hirai (Ed.). Phytopath. Soc. Japan, Tokyo.

38. Turel, F.L.M. 1969. Saprophytic development of the flax rust Melampsora lini, race no. 3. Can. J. Bot. 47: 821-823.

39. Turel, F.L.M. and G.A. Ledingham. 1957. Production of aerial mycelium and urediospores by Melampsora lini (Pers.) Lev on flax leaves in tissue culture. Can. J. Microbiol. 3: 813-819.

40. Turel, F.L.M. and G.A. Ledingham. 1959. Utilization of labelled substrates by the mycelium and urediospores of the flax rust fungus. Can. J. Microbiol. 5: 537-545.

41. Williams, P.G., K.J. Scott and J.L. Kuhl. 1966. Vegetative growth of Puccinia graminis f. sp. tritici in vitro. Phytopathology 56: 1415-1419.

42. Williams, P.G., K.J. Scott, J.L. Kuhl and D.J. Maclean. 1967. Sporulation and pathogenicity of Puccinia graminis f. sp. tritici grown on artificial medium. Phytopathology 57: 326-327.

43. Williams, P.G., and M. Shaw. 1968. Metabolism of glucose-C^{14}, pyruvate-C^{14}, and mannitol-C^{14} by Melampsora lini. I. Uptake. Can. J. Bot. 46: 435-440.

44. Wolf, F.T. 1974. The cultivation of rust fungi upon artificial media. Can. J. Bot. 52: 767-772.

45. Yarwood, C.E. 1956. Obligate parasitism. Ann. Rev. Plant Physiology 7: 115-142.

SECTION I

EXPERIMENTS USING PROTOPLASTS TO STUDY HOST:PARASITE INTERACTIONS

Chapter I

Development and Division of Protoplasts from Flax Cotyledons

INTRODUCTION

Isolated or naked protoplasts, as they are sometimes called, are cells from which the cell wall has been removed. Usually, removal is by digestion with enzymes although mechanical methods can also be used. Once the constraints of the cell wall disappear, the protoplast must be osmotically stabilized in solution to prevent death resulting from either bursting or contraction. Unlike normal plant cells, protoplasts are spherical. Each protoplast is independent of others and they are not connected by plasmadesmata. Although protoplast suspensions can be centrifuged at low speed and pipetted, they must be handled gently because they are extremely fragile and are easily burst.

Plant protoplasts have been shown capable of taking up macromolecules, organelles and bacteria (1, 8, 15, 17, 18, 19, 30), and have been used to obtain parasexual hybrids (5) and disease resistant mutants (4). Their isolation and development has been reviewed by Cocking (6). Great interest has been shown in the use of plant protoplasts in new techniques of crop improvement. Gamborg (11) has listed 9 species in

which protoplasts prepared from cultured cells have been shown to be capable of sustained cell division. Thus far, protoplasts derived from intact plants of three species have been shown to be capable of sustained division, tobacco (19, 27), petunia (2, 10), and pea (7); but only with tobacco have complete plants been grown from protoplasts isolated directly from leaves. Carlson (3) has pointed out that the use of protoplasts derived directly from intact plants avoids the problem of genetic variability inherent in the use of protoplast preparations derived from cell cultures. It is therefore important to attempt to increase the number of species from which protoplasts derived directly from intact plants can be induced to divide in culture and ultimately to grow into complete plants. This chapter describes attempts to grow protoplasts derived directly from fully expanded flax cotyledons.

MATERIALS AND METHODS

Fully expanded cotyledons were excised from flax seedlings (Linum usitatissimum L., varieties Bison and Bombay) after 2 to 4 leaves had developed (17°C, 2560 lux). They were washed in distilled water for 20 minutes, in 70% ethanol for 1 minute, 1% sodium hypochlorite for 20 minutes and then in sterile distilled water until used (maximum 20 minutes). The abaxial epidermis was removed and about

15 cotyledons incubated in 2 ml of enzyme solution for 18 h at 22°C.

The enzyme solution contained B5 medium (12) minus sucrose and hormones, 0.05 M glucose, 0.3 M sorbitol plus 2.5% cellulase (Onozuka P-5000), 2.5% mascerozyme (both from All Japan Biochemicals), 0.5% pectinase (Sigma) and 100 units of streptomycin/penicillin mixture. After digestion, vessel elements and epidermis were removed and the protoplasts washed free of enzyme by filtration (13). The wash medium contained B5 medium minus sucrose, 0.05 M glucose, 0.4 M sorbitol, plus auxin or cytokinin (see results) depending on the treatment. The washed protoplasts (5-10 ml) were then either mixed with an equal volume of wash medium plus agar (final agar concentration 0.5%) and spread in a thin film (0.5 mm) in a petri dish (35 mm) or with an equal volume of wash medium without agar and incubated as 0.1 ml droplets in petri dishes. The final concentration of protoplasts was 10^4/ml in both treatments. The petri dishes were then sealed with Parafilm and kept at high humidity in a plastic box at 22°C.

RESULTS AND DISCUSSION

Digestion at 37°C decreased the time required for protoplast isolation to about eight hours but resulted in a smaller proportion of living protoplasts (40%) than digestion at 22°C (75-80%). Satisfactory protoplast production occurred in

digestion media ranging in concentration from 0.15 M to 0.7 M (sorbitol plus glucose). Adjustment of the concentration of the wash medium to 0.1 M greater than that of the digestion medium increased the percentage of living protoplasts after washing. Although there are reports of deleterious effects from prolonged exposure of protoplasts to the digestive enzymes we did not observe this effect. Even after one month, protoplasts which had been left in the digestion medium were streaming vigorously.

In both liquid and agar treatments 75 to 80% of the protoplasts were intact at the beginning of incubation. Immediately after isolation the chloroplasts were often concentrated in the vicinity of the nucleus but were always at the periphery of the spherical protoplasts (Fig. I-1). Some of the larger, chloroplast-free protoplasts presumably originated from epidermal cells (see ref. 9). By day two, 15% of the protoplasts had died. The remainder doubled in size, their chloroplasts becoming evenly distributed about the periphery of each cell and cytoplasmic strands and streaming becoming visible. By day four many protoplasts had become elliptical or egg-shaped and "budding" (Fig. I-3) and division (Fig. I-2) were first observed. The percentage of divided cells increased to a maximum frequency of 0.4% on day 20. Both the cells resulting from divisions contained chloroplasts, which often became concentrated near the cross wall. Although cyclosis was observed in both cells

Figure I-1. Freshly isolated protoplasts. Day 1. X 260.

Figure I-2. Divided protoplast. Day 5. X500.

Figure I-3. Budding protoplast. Day 7. X500.

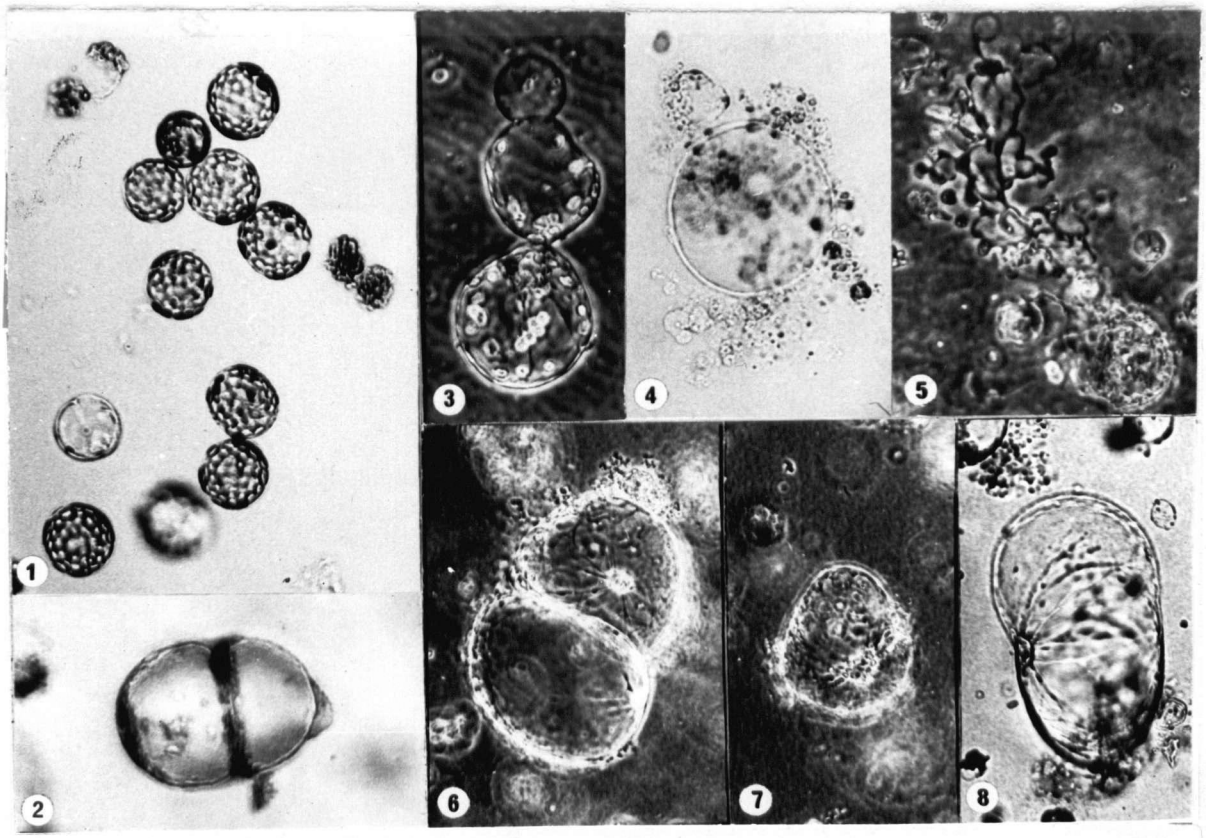
Figure I-4. Extrusion of cytoplasm, type I. Day 15. X600.

Figure I-5. Extrusion of cytoplasm, type II. Day 15. X525.

Figure I-6. Divided giant cell. Day 28. X430.

Figure I-7. Binucleate giant cell. Day 28. X260.

Figure I-8. Typical giant cell. Day 28. X260.


1
2
3
4
5
6
7
8

after division no multicellular aggregates were observed. Budding was observed with a frequency of 1% in agar cultures and 15% in liquid cultures. Extrusion of protoplast conents, a process akin to budding, was common in agar cultures. Two types of extrusion occurred (Figs. I-4 & I-5), the first type occurred about day 5. The extruded material was enclosed by a membrane and sometimes contained chloroplasts. In the second type, which gradually increased in frequency beginning on day 5, the extruded material was less organized and often originated from more than one area on the protoplast surface.

About 5% of the protoplasts began a second increase in size about day 20, by which time the chloroplasts had degenerated. Many of these protoplasts grew to ten times the diameter of freshly isolated protoplasts, contained enlarged nuclei and exhibited vigorous cytoplasmic streaming. Division of these cells was also observed (Fig. I-6).

Death of the cells originating from protoplasts occurred gradually from day 10 onwards and by day 40 nearly all were dead.

A number of conditions found to be suitable for development and division of protoplasts from other species were unsuitable for flax. Incubation in the light caused rapid death as did a medium successfully used with tobacco (14). No differences were observed between media which contained sucrose, glucose or ribose alone or in combination. Combinations of 2,4-dichlorophenoxyacetic acid, naphthalene acetic

acid, kinetin or benzyl amino purine ranging in concentration from 0.1 to 10 mg/l had no noticeable effects. Variation in the concentration of B5 medium from 0.1 to full strength, use of cotyledons of different ages, use of mannitol either alone or in combination with sorbitol, and variation of the molarity of the media also had no noticeable effect on protoplast behaviour. The medium successfully used for haploid petunia protoplasts by Binding (2) promoted the growth of increased numbers of giant cells (Figs. I-7, I-8) but did not increase the percentage of protoplasts which divided. If the concentration of protoplasts was less than 1×10^3/ml they failed to develop and died within a week; if greater than 1×10^5/ml, budding in liquid cultures and extrusion of cell contents were increased several fold. The responses of the two varieties Bison and Bombay to the various treatments was identical.

Although it was not possible to induce multiple cell divisions and the formation of callus from cultured flax protoplasts, 0.4% divided once. This rate of division is comparable to that obtained by Constabel et al. (7) with protoplasts isolated from pea stem tips. Both with flax and pea protoplasts the rate of division is much lower than has been achieved with tobacco protoplasts. Takebe et al. (21) were able to induce division in 50% of their tobacco protoplasts.

Flax cotyledons were found to be a good tissue from which to isolate protoplasts. Nearly 100% of the cotyledon cells were converted to protoplasts and they were easily washed and separated from undigested debris. Some protoplasts were killed during the isolation and washing procedure, however, preparations containing 75% to 80% living and healthy protoplasts were routinely prepared.

REFERENCES

1. Aoki, S., and I. Takebe. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. Virology 39: 439-449.

2. Binding, H. 1974. Cell cluster formation by leaf protoplasts from axenic cultures of haploid Petunia hybrida L. Plant Sciences Letters 2: 185-188.

3. Carlson, P.S. 1973. The use of protoplasts for genetic research. Proc. Nat. Acad. Sci. (U.S.A.) 70: 598-602.

4. Carlson, P.S. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180: 1366-1368.

5. Carlson, P.S., H.H. Smith and R.D. Dearing. 1972. Parasexual interspecific plant hybridization. Proc. Nat. Acad. Sci. (U.S.A.) 69: 2292-2294.

6. Cocking, E.C. 1972. Plant cell protoplasts - isolation and development. Ann. Rev. Plant Physiology 23: 24-50.

7. Constabel, F., J.W. Kirkpatrick and O.L. Gamborg. 1973. Callus formation from mesophyll protoplasts of Pisum sativum. Can. J. Bot. 51: 2105-2106.

8. Davey, M.R., and E.C. Cocking. 1972. Uptake of bacteria by isolated higher plant protoplasts. Nature 239: 455-456.

9. Davey, M.R., E.M. Frearson, L.A. Withers and J.B. Powers. 1973. Observations on the morphology, ultrastructure and regeneration of tobacco leaf epidermal protoplasts. Plant Science Letters 2: 23-27.

10. Durant, J., I. Potrykus and G. Donn. 1973. Plantes issues de protoplastes de Petunia. Z. Pflanzenphysiol. 69: 26-34.

11. Gamborg, O.L. and R.A. Miller. 1973. Isolation, culture and uses of plant protoplasts. Can. J. Bot. 51: 1795-1799.

12. Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Expt. Cell Res. 50: 151-158.

13. Kao, K.N., O.L. Gamborg, R.A. Miller and W.A. Keller. 1971. Cell division in cells regenerated from protoplasts of soybean and Haplopappus gracilis. Nature 232: 124.

14. Nagata, T., and I. Takebe. 1971. Plating of isolated mesophyll protoplasts on agar medium. Planta 99: 12-20.

15. Ohyama, K., O.L. Gamborg and R.A. Miller. 1972. Uptake of exogenous DNA by plant protoplasts. Can. J. Bot. 50: 2077-2080.

16. Ohyama, K., and J.P. Nitsch. 1972. Flowering haploid plants obtained from protoplasts of tobacco leaves. Plant and Cell Physiol. 13: 229-236.

17. Otsuki, Y. and I. Takebe. 1973. Infection of tobacco mesophyll protoplasts by cucumber mosaic virus. Virology 52: 433-438.

18. Otsuki, Y., T. Shimomma, and I. Takebe. 1972. Tobacco mosaic virus multiplication and expression of the N gene in necrotic responding tobacco varieties. Virology 50: 45-50.

19. Potrykus, I. 1973. Transplantation of chloroplasts into protoplasts of petunia. Z. fur Pflanzenphysiologie 70: 364-366.

20. Potrykus, I. and F. Hoffmann. 1973. Transplantation of nucleic acids into protoplasts of higher plants. Z. Pflanzenphysiologie 69: 287-289.

21. Takebe, I., G. Labib, and G. Melchers. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58: 318-320.

Chapter II

Experiments using a Protoplast Bioassay to Study Flax:Flax-Rust Interaction

INTRODUCTION

The extremely delicate nature of plant cell protoplasts renders them particularly sensitive to chemical stimuli or changes in their environment. The absence of a cell wall allows readily visible expansion and bursting of the protoplast to occur when protoplast integrity is disrupted by loss of function or structure of the plasmalemma, by increases in cell volume, or by disruptions within the cell such as disintegration of vacuoles. Thus toxins or other compounds which influence protoplast integrity and function can be detected by a bioassay using protoplasts.

The bursting response of protoplasts has been used as the basis of bioassays before. Ruesink (19, 20) incubated protoplasts derived from Avena coleoptiles with a number of compounds in an investigation of plasmalemma structure. Avena protoplasts burst when incubated in solutions containing anionic detergents (sodium dodecyl sulfate, and taurocholic acid) a cationic detergent (hexadecyl trimethyl ammonium bromide) and a non-ionic detergent (Triton X-100 but not Tween 80). The basic proteins, RNase and cytochrome

C also caused bursting but trypsin did not. Proteolytic and lipolytic enzymes as well as peroxidase and polyphenyl oxidase also failed to cause bursting. Ruesink concluded from evidence obtained with his protoplast bioassay that tyrosine plays no important role in the Avena plasma membrane in contrast to some animal cell membranes where it does.

Ruesink was unable to demonstrate a bursting response of Avena protoplasts to indoleacetic acid (IAA) although IAA is known to cause bursting of protoplasts obtained from other species (2). Cocking (5, 6, 7) used a tomato root protoplast bioassay to investigate compounds known to have effects on plant growth. Indole acetic acid, naphthaleneacetic acid (NAA), gibberellic acid, ethylenediaminetetracetic acid, 8-hydroxyquinoline and benzylpenicillin all increased vacuolation of protoplasts and caused them to burst. No firm conclusions could be drawn from this work.

Boulware and Camper (3) studied the effects of several herbicides on the bursting response of protoplasts. Although paraquat caused bursting, preforan, fluometuran, chlorbromuron and trifluralin did not. The study was undertaken to contribute to the understanding of the mode of action of these pesticides. In contrast to the results of Ruesink and Thimann (20), Boulware and Camper (3) found Tween 80 to be toxic to tomato fruit protoplasts.

Protoplasts and cells isolated from leaves have been

used in several studies investigating host:parasite interactions. Strobel and Hess (22), studying the fungal toxin Helminthosporicide produced by the sugarcane pathogen Helminthosporium sacchari, incubated protoplasts obtained from susceptible and resistant varieties in a solution containing the toxin. The hypothesis that the toxin binds to a specific protein located on the plasmalemma was supported by the fact that only protoplasts from the susceptible variety burst. Four hours of incubation were required to demonstrate the effect. Strobel and Hess (22) found that 5.0 mM toxin was required to burst isolated protoplasts, whereas only 0.01 μM was required to cause symptoms in an 8 cm leaf piece. The large difference in concentration between that required to burst protoplasts compared to that which caused leaf symptoms suggests that something other than loss of plasmalemma function is involved in symptom development.

Samaddar and Scheffer (21) studied the effects of Helminthosporium victoriae toxin on protoplasts prepared from coleoptiles of both resistant and susceptible oat varieties as well as from corn and sorghum. The protoplasts were incubated with toxin at a concentration of 0.16 μg/ml. Complete inhibition of root growth of susceptible oat varieties occurs at a toxin concentration of 0.0016 μg/ml. After 1 h incubation in the toxin solution, 100% of the protoplasts from the susceptible oat variety had lysed. No bursting of either the resistant oat protoplasts or those prepared from corn

or sorghum occurred. If the toxin solution was diluted, the protoplasts from the susceptible oat variety took longer to burst. Samaddar and Scheffer (21) concluded from these results that membrane effects are very important in the action of this toxin and that the action of Helminthosporium victoriae toxin on single protoplasts could be observed.

Protoplasts have also been used in a study by Otsuki et al. (16) in which the necrotic response of tobacco to tobacco mosaic virus was studied. They found that although TMV multiplied in protoplasts of both necrotic and systemic responding varieties, no differences between the two types of protoplasts were observable. From this observation they concluded that necrosis is a manifestation requiring organized tissue as in an intact leaf. The results of Strobel and Hess (22) and Otsuki et al. (16) both suggest that the disease reaction of isolated protoplasts is different from that of the intact plant.

Protoplasts have not previously been used to study host:rust interactions. Although I recognized that the results might not be directly comparable with the effects of the fungus on an intact tissue, which is composed of many contiguous cells, I considered that it would be valuable to examine the early interactions between isolated host protoplasts and the rust fungus. The early interaction between obligate fungal parasites and their hosts is an area of investigation of obvious importance, but has been

largely neglected. The chief reason for this neglect is the difficulty of investigating a stage of infection in which the number of host cells affected is a very small proportion of the total number in the infected leaf. An important advantage of the protoplast bioassay is that any effects of the fungus on its products would be exerted in a synchronized manner on the protoplast population in the bioassay medium.

If a bursting response occurred it would be a sensitive and easily observed measure of the interaction, particularly if a toxin were involved. Several observations suggested that this might be the case. Caldwell and Stone (1936) observed that guard cells are killed when a germinating rust spore grows through them. Littlefield (13) has pointed out that the necrosis of cells immediately adjacent to an incipient rust infection in flax, varies in extent and character depending on the genotype of both the host and rust. Necrotic areas around wheat rust pustules can be increased in size and extended away from the infected area of the leaf by the manipulation of an electrical field (15). The necrotic area extends towards the positive pole when a current of 2-4 microamperes was applied to the infected leaf.

The following experiments were therefore designed to investigate interaction between flax rust exudate and flax protoplasts prepared from both resistant and susceptible varieties. Bursting of protoplasts when incubated with rust

exudate would indicate the presence of (a) fungal toxin(s) and would support the toxin theory of resistance and susceptibility.

MATERIALS AND METHODS

The isolation and washing of protoplasts is described in Chapter I. The protoplasts used in these experiments were prepared in an identical manner. The protoplasts were used within 2 h after washing. Two varieties of flax were used: Bison (susceptible) and Bombay (resistant).

Spore germination medium was prepared by germinating 20 mg of flax rust spores (Melampsora lini, race 3) on the surface of 1 ml of 0.35M sorbitol spread out into a thin layer in a petri dish. After overnight germination (the percent germination was greater than 50%) the spores and liquid were transferred to a tapered centrifuge tube. They were then shaken in a Vortex mixer, allowed to sit for 0.5 h, then shaken again and centrifuged to sediment the spores. The supernatant was the "spore germination medium". Exudate from axenically cultured flax rust (race 3) was prepared in a similar manner except that 1 gm (fresh weight) of rust was incubated overnight in 2 ml of 0.35 M sorbitol. This is referred to as "axenic exudate".

To assay the percentage of living protoplasts a suspension of 250 or more protoplasts in 0.1 ml of 0.35 M sorbitol was placed on a depression slide. The number of both living

and dead protoplasts were counted using a microscope with phase contrast optics. The test or control solution was added and the percentage of living protoplasts calculated by counting the protoplasts on each slide. This took about 10 minutes. Consecutive counts indicated that the variation between counts was 10%. Viability of protoplasts was easily determined. A smooth outline of the protoplasts, streaming, the central position of the vacuole and evenly distributed chloroplasts in the cytoplasm were all indicative of healthy protoplasts.

RESULTS

Different preparations of protoplasts were consistent in appearance. Protoplasts were prepared from the two varieties Bison and Bombay. In each case 60 to 80% were alive and in an apparently healthy state after washing and at the beginning of experiments. There was some morphological variation amongst the viable protoplasts. For example, in some, the chloroplasts were evenly spaced around the periphery of the cell but in others they were concentrated on one side usually in clusters around the nucleus.

In attempting to make the protoplast bursting bioassay as sensitive and consistent as possible, a number of procedural variations were investigated and different sources of fungal material including germinating spores and axenic cultures were used. Embedding the protoplasts in a thin layer of agar (0.5 mm) and applying the test solution by flooding the surface had

the advantage of immobilizing the protoplasts. This method was, however, discarded because the presence of agar decreased the visibility and made it more difficult to determine viability. The agar also caused a marked increased in the rate of death of both treated and control protoplasts during the first hours of the experiment. Varying the concentration of protoplasts, although it had an effect on the growth and development of protoplasts incubated for periods of up to a month (see Chapter I), did not have an observable effect during the incubation periods of up to 20 h used in these experiments. About 300 protoplasts in 0.2 ml of solution could be counted easily and quickly (10 minutes). Sealing the protoplasts in a micro-incubation chamber made of coverslips prevented evaporation during the 10 minutes required for counting. It was so cumbersome and time-consuming, however, that it was abandoned. Evaporation from depression slides was not a problem if the slides were kept in a petri dish containing moist filter paper except during the counting procedure.

Table I summarizes the results of an experiment in which spore germination medium plus spores was used as the test solution. In the control solution the protoplasts were incubated in an equal volume of 0.35 M sorbitol. Each figure in Table I is an average of 2 separate counts. The results show that in this experiment treatment with the spore germination medium plus spores did not cause protoplast bursting; if anything, a protective effect is suggested, the decrease

TABLE I. Effects of Spore Germination Medium Plus Spores on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	0.5	1.0	1.5	2.0	17.0	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]								
Bison	68.7	-	66.0	-	57.5	63.4	5.2	
Bombay	70.3	-	61.6	-	57.4	54.3	16.0	
TREATED[5]								
Bison	61.9	58.7	63.4	58.4	56.4	57.3	4.6	+0.6
Bombay	67.3	59.7	52.7	48.8	53.6	59.5	7.8	+8.2

[1]Observed decrease.
[2]Observed decrease corrected for control decrease of the same variety.
[3]Hours from start of experiment.
[4]Protoplasts (1 part) plus 0.35 M sorbitol (9 parts).
[5]Protoplasts (1 part) spore germination medium plus spores (9 parts).

in the percentage of living protoplasts being 8.2% less in the treated than in the corresponding control. The protective effect of the treatment on Bombay protoplasts is probably not real, because the percentage of living protoplasts at 1.5 h was 11% less than at 17 h, an impossibility and a reflection of the variability in counting.

The results of a similar experiment are presented in Table II. In this case the spores were germinated overnight rather than for 2 h. The results suggest a possible effect of the treatment in increasing bursting, but there is no difference between the susceptible variety Bison and the resistant Bombay. There is little, if any, evidence of inconsistency in counting apparent in this experiment.

In the experiment summarized in Table III both protoplast isolation and the bioassay were conducted at 37°C for 8 h, instead of at 22°C overnight as was the usual procedure. The bursting response was measured at 37°C because this temperature was known to be higher than the optimal temperature for flax protoplast growth and development. The possibility was considered that the protoplasts would be more sensitive at the higher temperature if the kinetics of toxin action (assuming a toxin was present) were characterized by a high Q_{10}. The treatment consisted of germinated spore medium adjusted to 0.35 M with respect to sorbitol and did not contain spores. The spores had been germinated overnight and then processed as described in the methods section.

TABLE II. Effects of Spore Germination Medium Plus Spores on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	1.0	1.5	3.0	18.0	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]							
Bison	63.7	63.3	48.7	56.1	57.7	6.0	
Bombay	67.7	64.8	65.9	65.4	58.4	9.3	
TREATED[5]							
Bison	61.5	56.6	56.0	44.8	45.2	16.3	-10.3
Bombay	59.3	50.0	48.2	46.2	43.7	16.6	- 7.3

[1]Observed decrease.

[2]Observed decrease corrected for the control decrease of the same variety.

[3]Hours from start of experiment

[4]Protoplasts (1 part) plus 0.35 M sorbitol (9 parts).

[5]Protoplasts (1 part) plus spore germination medium plus spores (9 parts).

TABLE III. Effect of 37°C Incubation on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	1.5	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]				
Bison	52.7	34.9	17.8	
Bombay	49.9	33.2	16.7	
TREATMENT 1[5]				
Bison	49.6	26.9	22.7	-4.9
Bombay	52.2	27.2	25.0	-8.3
TREATMENT 2[6]				
Bison	42.8	16.3	26.5	-8.7
Bombay	51.4	29.0	22.9	-5.7

[1]Observed decrease.

[2]Observed decrease corrected for the control decrease of the same variety.

[3]Hours from start of experiment.

[4]Protoplasts (1 part) plus 0.35 M sorbitol (10 parts).

[5]Protoplasts (1 part) plus spore germination medium (10 parts).

[6]Protoplasts (1 part) plus spore germination medium (1 part) plus 0.35 M sorbitol (9 parts).

As can be seen from the results (Table III), the percentage of living protoplasts after washing was reduced by raising the temperature during isolation. The rate of death of both the control and the treated protoplasts was also increased during the experiment. The increase in temperature was not effective in increasing the difference between the controls and the two treatments.

Tables IV and V are two experiments in which spore germination medium was tested. The two experiments were identical in all respects, except that they were not carried out on the same day. They sum up the difficulties which were encountered in demonstrating consistent and reliable evidence for the existence of a toxin of fungal origin. Although there was an apparent treatment effect in both experiments this is seen to be inconsistent. For example, in Table IV, treatment 1 (high concentration of spore germination medium), the decrease in protoplast viability is the same as in the control. In Table V, treatment 1, the decrease in protoplast viability was 10-15%. Examination of the results for treatment 2 in Tables IV and V (dilute spore germination medium) shows an effect which is the reverse of that seen for treatment 1. In Table IV the dilute spore germination medium decreased protoplast viability 7 to 12% but in Table V it was the same as in the control. The results presented in Tables VI and VII are from two identical experiments which were carried out on different

TABLE IV. Effect of Spore Germination Medium on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	1.0	2.5	16	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]						
Bison	77.6	75.7	73.1	69.2	8.4	
Bombay	79.5	81.5	72.6	71.2	8.3	
TREATMENT 1[5]						
Bison	71.3	68.4	64.8	61.1	10.2	- 1.8
Bombay	74.9	72.1	65.7	69.7	5.2	+ 3.1
TREATMENT 2[6]						
Bison	70.2	68.1	65.8	59.7	20.5	-12.1
Bombay	76.6	72.7	67.0	61.4	15.2	- 6.9

[1]Observed decrease.

[2]Observed decrease corrected for the decrease of the control of the same variety.

[3]Hours from start of experiment.

[4]Protoplasts (1 part) plus 0.35 M sorbitol.

[5]Protoplasts (1 part) plus spore germination medium (10 parts).

[6]Protoplasts (1 part) plus spore germination medium (1 part) plus 0.35 M sorbitol (9 parts).

TABLE V. Effects of Spore Germination Medium on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	0.5	2.0	3.0	20	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]							
Bison	81.6	78.1	77.0	71.7	74.6	7.0	
Bombay	62.7	67.6	61.3	57.9	55.7	7.0	
TREATMENT 1[5]							
Bison	68.7	67.8	62.4	64.0	43.1	17.2	-10.2
Bombay	66.1	74.0	71.4	68.5	51.5	23.0	-16.0
TREATMENT 2[6]							
Bison	71.7	70.6	68.8	64.4	66.8	4.9	+ 2.1
Bombay	73.0	73.4	70.3	68.6	66.2	6.2	- 0.8

[1]Observed decrease.

[2]Observed decrease corrected for the decrease of the control of the same variety.

[3]Hours from start of experiment.

[4]Protoplasts (1 part) plus 0.35 M sorbitol (10 parts).

[5]Protoplasts (1 part) plus spore germination medium (10 parts).

[6]Protoplasts (1 part) plus spore germination medium (1 part) plus 0.35 M sorbitol (9 parts).

days. The protoplast suspensions were incubated with axenic exudate at two different concentrations (treatment 1 and 2). Although the results in Table VI suggest that the exudate obtained from axenic cultures causes more bursting than the exudate from germinating spores the effects of the treatment (Table VI, treatment 1) is still not conclusive. Thus in the experiment shown in Table VII the effect of treatment 1 on protoplasts from the two different varieties of flax is not as pronounced as in the experiments in Table VI. Moreover, protoplasts from Bombay flax which were the most susceptible to bursting in Table VI, were highly resistant to the treatment in the experiment in Table VII. Finally, there was in these experiments (Tables VI and VII) no consistent difference in the response of the two varieties, Bison and Bombay.

DISCUSSION

In the preceding experiments which are described in Tables I to VII the protoplast bioassay was shown to be useful and the counting of living protoplasts to be consistent. Preliminary experiments designed to test the consistency of counting the percentage of living protoplasts showed that when consecutive counts of the same slide were made the variability in the percentage of living protoplasts was 10%.

In the experiments described in Tables I to VII, the variability of counting living protoplast was also usually

TABLE VI. Effects of Axenic Exudate on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	1.0	2.5	21	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]						
Bison	87.1	84.1	81.3	74.5	12.6	
Bombay	73.5	72.2	65.7	64.8	8.7	
TREATMENT 1[5]						
Bison	80.0	76.0	74.4	51.4	28.6	-16.0
Bombay	75.8	73.3	69.7	47.7	28.1	-19.4
TREATMENT 2[6]						
Bison	82.9	78.7	76.9	72.7	10.2	- 2.4
Bombay	82.5	75.5	72.6	72.6	9.9	+ 1.2

[1]Observed decrease.

[2]Observed decrease corrected for the decrease of the control of the same variety.

[3]Hours from start of experiment.

[4]Protoplasts (1 part) plus 0.35 M sorbitol (10 parts).

[5]Protoplasts (1 part) plus axenic exudate (10 parts).

[6]Protoplasts (1 part) plux axenic exudate (1 part) plus 0.35 M sorbitol (9 parts).

TABLE VII. Effects of Axenic Exudate on Protoplasts

HOURS[3]	LIVING PROTOPLASTS(%) 0	1.0	1.5	2.5	17	CHANGE IN PROTOPLAST VIABILITY(%) A[1]	B[2]
CONTROL[4]							
Bison	84	74	76	74	70	14	
Bombay	83	79	80	70	68	15	
TREATMENT[5]							
Bison 1	83	68	63	68	58	25	-11
Bison 2	83	80	69	72	64	19	- 5
Bombay 1	84	66	68	65	66	18	- 2.5
Bombay 2	83	69	71	68	68	15	+ 0.5

[1]Observed decrease.

[2]Observed decrease corrected for the decrease of the same control variety.

[3]Hours from start of experiment.

[4]Protoplasts (1 part) plus 0.35 M sorbitol (10 parts).

[5]Protoplasts (1 part) plus axenic exudate (10 parts).
1 and 2 are replicates of the same experiment.

within this range. If inconsistency of counting had occurred the percentage of living protoplasts would sometimes increase, rather than decrease or remain the same, as consecutive counts of the slide were made during the experimental incubation period. The greatest inconsistency of this kind was in the variety Bombay, Table I, treatment 1. Here, the percentage living protoplasts was 48.8% after 1.5 h but at 17 h was 59.5%, an inconsistency of 10.7%.

Another measure of the consistency of counting percentage living protoplasts is a comparison of protoplasts of the same variety, in the same experiment at 0 h. Since all the protoplasts from each variety were from the same stock suspension these counts should not vary more than 10%. Examination of Tables I-VII shows that a variation of greater than 10% only occurred in Table V. At 0 h Bison protoplasts ranged in percentage living protoplasts from 81.6% to 68.7%, a difference of 12.9% and Bombay protoplasts ranged from 62.7% to 73.0%, a difference of 11.0%.

In nearly all the experiments (Tables I to VII), the protoplasts from Bison and Bombay flax varieties when incubated with either spore germination medium or axenic exudate burst to a greater extent than those in the corresponding control solution. The decrease, when corrected for the control, was usually less than 10%. In the experiments presented in Tables V and VI the treatment decrease after correction for the control decrease was greater than 10%. As has been

argued above, this apparent effect of spore germination medium (Table V) and axenic exudate (Table VI) is not real. It could not be demonstrated in identical experiments carried out on different days. There is even less evidence that the two varieties Bison and Bombay, responded differently to any of the treatment solutions.

An additional analysis of the results, using statistical techniques is presented in the appendix at the end of this chapter.

From the discussion, therefore, it can be concluded that although the bioassay technique is reliable, no toxic factor either from germinated spores or axenic cultures was detected. At least two possibilities exist. Either a toxic substance was present at low concentration, but was not detected because of the 10% variability in counting viable protoplasts or, there was no toxic substance present.

What then is the explanation of the observations which prompted the search for toxic factors produced by the fungus? It seems apparent that the observations which suggested the involvement of a toxic substance result from processes that only occur when the rust interacts with the intact plant. Otsuki et al. (16) concluded from their experiments with virus infected protoplasts from necrotic and susceptible responding tobacco that organization of cells, as in the intact leaf, is necessary for the necrotic response. This also seems to be the case with flax rust and flax. Since the spores and axenic cultures

used in experiments are known to be pathogenic but did not cause protoplasts to burst, the explanation of necrosis during disease may be that the toxic factors causing necrosis originate from plant cells rather than from the fungus. A good possibility is that these toxic factors of plant origin are phytoalexins or compounds which act in a similar manner. Phytoalexins are produced by plant cells and are rarely found except in association with necrosis (14). Their production can be induced by a wide range of pathogens as well as by wounding (17). Both their production and necrosis are non-specific responses in contrast to susceptibility and resistance. In the flax:flax rust system resistance and susceptibility are dependent on one gene in the fungus and one in the host (9). The results support the idea that resistance is the result of a process which is distinct and different from those causing the necrotic reaction. This is a new concept and one which is important to the understanding of resistance and susceptibility of plants to pathogens. A very similar proposal has recently been advanced by Kiraly et al. (11). These authors suggest that hypersensitivity is the result and not the cause of resistance and have presented evidence that resistance can be expressed without the occurrence of necrosis (10).

No compounds with phytoalexin-like properties have been described from infected flax although many phenolic compounds are known to occur in flax. In the wheat:wheat rust system

although phytoalexins *per se* are not thought to be produced other toxic phenolic compounds which may have many similar properties have been identified (8). The phenolic glycoside of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (12) is present in wheat plants and is known to break down into the aglycone and benzoxazoline upon infection of wheat by an avirulent race of rust. Both of these breakdown products are known to have considerable antifungal activity. Like phytoalexin production, breakdown of the glycoside is not gene specific.

If phytoalexin production and the associated necrosis is the result of resistance, what is the nature of the specific interaction between the pathogen and the plant which results in susceptibility or resistance? There is very little experimental information on this subject but several proposals have been made. Albersheim *et al.* (1) put forward an interesting hypothesis along with a convincing discussion and explanation. They consider

> "That molecular interactions between the carbohydrate constituents of a host and the polysaccharide degrading enzymes produced by a pathogen account for the inherent resistance of plants to most microorganisms and that these interactions account equally well for the rare instances in which a microorganism successfully infects a plant."

If this hypothesis is essentially correct, then susceptibility and resistance would not be expected to occur or be observed in a system in which pathogens interact with protoplasts because protoplasts do not have cell walls. The results of the

experiments presented in this report are therefore consistent with Albersheim's hypothesis but it would nevertheless be difficult to argue that they provide any degree of proof of it.

A second hypothesis is that a specific gene product is produced by the fungus which when in interaction with plant cells determines susceptibility or resistance. In a recent report Rohringer et al. (18) claim to have isolated a gene-specific RNA directly involved in resistance of wheat to wheat rust from heavily infected and necrotic wheat leaves. Their bioassay involves injecting the RNA fraction into susceptible or resistant wheat leaves infected with rust and counting the numbers of necrotic cells in each case after 11 h. The RNA is active only in infected leaves containing haustoria. This finding and the conclusions drawn from the work remain to be confirmed.

No evidence which supports the specific gene product theory was obtained from the protoplast experiments reported here. If this theory is true, the spore and axenic culture extract could contain the "gene product" since the spores and axenic cultures are known to be pathogenic. The recognition of resistance or susceptibility would occur when the gene product interacted with the protoplasts. Protoplast bursting would not necessarily be expected to occur because, as discussed above, the necrotic response may only occur when there is an interaction of infected cells with surrounding tissues in an intact leaf.

REFERENCES

1. Albersheim, P., T.M. Jones and P.D. English. 1969. Biochemistry of the cell wall in relation to infection processes. Ann. Rev. Phytopathology 7: 171-194.

2. Boyer, M.H. 1973. Response of Nicotiana mesophyll protoplasts of normal and tumorous origin to indoleacetic acid in vitro. Plant Physiology 51: 898-901.

3. Boulware, M.A. and N.D. Camper. 1972. Effects of selected herbicides on plant protoplasts. Physiol. Plantarum 26: 313-317.

4. Caldwell, R.M., and G.M. Stone. 1936. Relation of stomatal function of wheat to invasion and infection by leaf rust Puccinia tritici. J. Agr. Res. 52: 917-932.

5. Cocking, E.C. 1961. Properties of isolated protoplasts. Nature 191: 780-782.

6. Cocking, E.C. 1962. The action of indolyl-3-acetic acid on isolated protoplasts of tomato cotyledons. Biochem. J. 82:

7. Cocking, E.C. 1962. Action of growth substances, chelating agents and antibiotics on isolated root protoplasts. Nature 193: 998-999.

8. ElNaghy, M.A. and M. Shaw. 1966. Correlation between resistance to stem rust and the concentration of a glucoside in wheat. Nature 210. 417-418.

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

10. Kiraly, Z. 1974. Tissue necrosis and plant disease resistance. Am. Phytopath. Soc./Can. Phytopath. Soc. Ann. Meeting 1974, Abstr. 165.

11. Kiraly, Z., B. Barna and T. Ersek. 1972. Hypersensitivity as a consequence, not the cause, of plant resistance to infection. Nature 239: 456-457.

12. Knott, D.R. and J. Kumar. 1972. Tests of the relationship between a specific phenolic glucoside and stem rust resistance in wheat. Physiol. Plant Pathology 2: 393-399.

13. Littlefield, L.J. 1973. Histological evidence for diverse mechanisms of resistance to flax rust, Melampsora lini (Ehrenb.) Lev. Physiol. Plant Pathology 3: 241-247.

14. Müller, K. and H. Berger. 1940. Experimentelle untersuchungen uber die Phytophthora resistenz der kartoffel. Arb. Biol. Reichsanstat Land-U Forstwritsch, Berlin 23: 189-231.

15. Olien, C.R. 1957. Electrophoretic displacement of the necrotic area from the region of mycelial development in Khapli emmer infected with race 56 of Puccinia graminis var. tritici.,, Phytopathology 45: 26 (Abstr.).

16. Otsuki, Y., T. Shimomura and I. Takebe. 1972. Tobacco mosaic virus multiplication and expression of the N gene in necrotic responding tobacco varieties. Virology 50: 45-50.

17. Rahe, J.E. 1974. Accumulation of phaseollin in hypocotyls of etiolated Phaseolus vulgaris in response to mechanical injury. Am. Phytopath. Soc./Can. Phytopath. Soc. 1974 Meeting. Abstr. 196.

18. Rohringer, R., N.K. Howes, W.K. Kim and D.J. Samborski. 1974. Evidence for a gene-specific RNA determining resistance in wheat to stem rust. Nature 249: 585-588.

19. Ruesink, A.W. 1971. The plasma membrane of Avena coleoptile protoplasts. Plant Physiology 47: 192-195.

20. Ruesink, A.W. and K.V. Thimann. 1965. Protoplasts from the Avena coleoptile. Proc. Nat. Acad. Sci. (U.S.A.) 54: 56-64.

21. Samaddar, K.R. and R.P. Scheffer. 1966. Effects of Helminthosporium victoriae toxin on protoplasts from Avena coleoptiles. Phytopathology 56: 898 (Abstr.).

22. Strobel, G.A. and W.M. Hess. 1974. Evidence for the presence of the toxin-binding protein on the plasma membrane of sugar cane cells. Proc. Nat. Acad. Sci. (U.S.A.) 71: 1413-1417.

APPENDIX TO CHAPTER II

A more detailed analysis of the results which are presented in Tables I-VII and discussed above is possible using statistical techniques. In particular, the possible effect on the bursting of protoplasts caused by spore germination medium and axenic exudate can be quantified in more precise terms and limits on these effects set.

Two analyses were carried out, the first on those experiments which included control protoplasts and one treatment (Tables I, II and VII) and the second which included two treatments plus the control (Tables IV, V and VI). The experiment presented in Table III was not included in either analysis because only two counts of the percentage living protoplasts were made (at 0 and 1.5 h).

In both the first and second analyses a randomized block design was used, the different experiments being designated as blocks. For the purposes of the analysis only the counts taken at four different times during the experiment were used. Thus in the experiment in Table I those counts made at 0.5 and 1.5 h were not used. Similarily in Table II those at 1.5 h, in Table VII those at 1.5 h and in Table V those at 2.0 h were not used. In the experiment in Table VII two slides for each variety were incubated with the treatment solution. The two slides for each variety were averaged to give one count for each variety. These adjustments made the design of the experiments similar so that the analysis could be done.

Analysis of Variances, Tables I, II and VII

Source	df	MS	F
Experiment	b-1 = 2	1236.67	80.87**
Treatment	a-1 = 1	446.52	29.20**
Variety	c-1 = 1	5.88	.38NS
Time	h-1 = 3	380.56	24.89**
Time X Variety	(a-1)(c-1) = 1	8.71	.53NS
Treatment X Time	(a-1)(h-1) = 3	9.75	.64NS
Variety X Time	(c-1)(h-1) = 3	13.42	.88NS
Treatment X Variety X Time	(a-1)(c-1)(h-1) = 3	26.89	1.75NS
Experimental Error	(b-1)(ach-1) = 30	15.29	-
Total	bach-1 = 47	-	-

** highly significant, $P < 0.01$
NS not significant, $P > 0.05$

The results of the analysis of variance for the data in Tables I, II and VII show that in these experiments the treated protoplasts burst to a significantly greater extent than those incubated in the control solution. The mean percentage of living protoplasts for the control was 66.60% and for the treated was 60.50%. A 95% confidence interval about the difference can be constructed as follows:

$$(\bar{X}_1 - \bar{X}_2) \pm t_{30df(1\text{-tailed})} \sqrt{\frac{EMS}{24} + \frac{EMS}{24}}$$

Numerically, the difference between the means including the 95% confidence interval calculated using the above equation is $6.10\% \pm 1.13\%$. The conclusions that can be drawn from the above analysis are that the decrease of percentage living protoplasts for the treated protoplasts over that of the control protoplasts was significant and that the difference (95% confidence interval) is between 4.18% and 8.01%.

Analysis of Variance, Tables IV, V and VI

Source	df	MS	F
Experiment	b-1 = 2	258.84	9.41**
Treatment	a-1 = 2	261.35	9.51**
Varieties	c-1 = 1	72.20	2.63NS
Time	h-1 = 3	555.94	20.22**
Treatment X Variety	(a-1)(c-1) = 2	198.54	7.22**
Treatment X Time	(a-1)(h-1) = 6	57.97	2.11NS
Variety X Time	(c-1)(h-1) = 3	6.86	0.25NS
Treatment X Variety X Time	(a-1)(c-1)(h-1) =6	5.19	0.19NS
Experimental Error	(b-1)(ach-1) = 46	27.49	-
Total	bach-1 = 71	-	-

** highly significant, $p < 0.01$
NS not significant, $P > 0.05$

The results of the analysis of variance for the data in Tables IV, V and VI are that in these experiments, as in those in Tables I, II and VII, the treated protoplasts burst to a significantly greater extent than those incubated in the control solution. In this analysis although the varieties did not respond significantly differently there was a variety X treatment interaction which shows that the nature of the responses of the two varieties was different.

The same method as that described above can be used to calculate the magnitude of the differences, with 95% confidence. The two varieties are dealt with separately because of the variety X treatment interaction. Bison control (mean 77.3%) was less than Bison, treatment 1, by the interval 7.87% to 15.06%. Bombay control (mean 69.0%) did not differ from Bombay, treatment 1 (-2.27% to 4.92%). Bison control was less than Bison, treatment 2, by the interval 3.08% to

10.27%. Bombay control did not differ from Bombay, treatment 2 (-6.69 to 0.49).

Although the results from the statistical analysis clearly show a greater decrease of percentage living protoplasts for the treated than for the control protoplasts, the magnitude of the difference is small, usually less than 10%. Samaddar and Scheffer (21) for example, observed 100% bursting of protoplasts after 1 h when they incubated protoplasts from a susceptible oat variety with Helminthosporium victoriae toxin. Even if the toxic substance in the treatment solutions was very dilute it would be expected that the difference between the control and treated protoplasts would be greater than 10% after 20 h incubation. The difference in the decline in the percentage of living protoplasts between treatment 1 and the dilute treatment 2 would also be expected to be greater.

There is at least one possible reason why a difference between treated and control protoplasts could occur even if no toxic substance was present, namely an unconscious psychological bias in the counting. This possibility exists because the identity of the control and treatment slides was known to the counter.

SECTION II

AXENIC CULTURE

Chapter III

Axenic Culture of Flax Rust Isolated from Cotyledons by Cell Wall Digestion*

INTRODUCTION

Otsuki and Takebe (8) isolated intact mesophyll cells and their protoplasts from a number of plant species by using mixtures of hydrolytic enzymes to digest the plant cell walls. They noted that some species were more resistant to the enzymes than others. Maheshwari (7) used a similar technique to isolate the epidermis of sunflower and snapdragon leaves but found it more difficult to obtain suitable preparations from wheat and corn.

A method is reported here for the isolation of intact colonies of the obligate parasite (Melampsora lini (Pers.) Lev., Race No. 3) from flax by the use of hydrolytic enzymes which selectively digest the cell walls of the host plant, without apparently damaging the fungal cell wall. Once isolated, the colonies may be useful for metabolic and other studies of the rust fungus in the absence of host tissue. Such colonies can also be used as inoculum for the establishment of axenic cultures.

---

*Published in Canadian Journal of Botany Vol. 50:2601-2603, 1972.

MATERIALS AND METHODS

Growth and inoculation of flax seedlings (variety Bison) and surface sterilization of the cotyledons were carried out as described by Coffey *et al.* (3). Cotyledons were harvested when infection flecks were well-developed but not yet orange in colour. After surface sterilization, the abaxial epidermis was stripped away as carefully as possible using fine forceps and a spatula. The cotyledons were then immersed in the B-5 medium described by Gamborg *et al.* (5), modified by the omission of 2,4-D and containing in addition 2.5% macerozyme and 2.5% Onozuka-P5000 cellulase (both obtained from All Japan Biochemicals), 0.5% pectinase (Sigma) and Gramicidin D (6 μg/ml). If a large yield of colonies was desired, about sixty cotyledons were immersed in 25 ml of the enzyme mixture in a 50 ml Erlenmyer flask, infiltrated under vacuum until they sank, and incubated for 90 minutes at room temperature with gentle shaking. This was followed by agitation with a magnetic stirrer for 30 minutes. The speed of the stirrer was adjusted to avoid foaming. The liberated colonies were now suspended in the medium and were collected on a nylon screen (385 μ). They were then washed with modified B-5 medium (i.e. minus 2,4-D), resuspended in modified B-5 medium and centrifuged at low speed to concentrate them in a loose pellet. This procedure is referred to as procedure I.

Procedure II was used to obtain colonies which were virtually free of chloroplasts and flax cells. About six cotyledons with the abaxial epidermis removed were incubated in a plastic petri dish (Falcon, 60 mm), containing 2 ml of enzyme solution, at room temperature overnight. The colonies were then freed from the epidermis by gentle shaking, transferred to about 25 ml of modified B-5 medium and agitated with a magnetic stirrer for 10 minutes. This suspension was poured into a petri dish. Colonies were spotted through a dissecting microscope and picked up with a Pasteur pipette. Axenic cultures were initiated by transferring the colonies, suspended in modified B-5 medium to the agar medium used for axenic culture of the rust. The droplet of liquid was then removed to ensure direct contact of the colonies with the agar surface. Each liter of the agar medium contained KNO_3, 0.25 gm; $Ca(NO_3)\cdot 4H_2O$, 2 gm; K_2HPO_4, 0.75 gm; KH_2PO_4, 0.25 gm; NH_4NO_3, 0.04 gm; the micronutrients described by Coffey and Shaw (3); sucrose, 40 gm; agar, 16 gm; and bovine serum albumin (Miles, Fraction V), 0.5 gm. The calcium nitrate was autoclaved separately and added to the medium when it had cooled. The bovine serum albumin was defatted (1) and filter sterilized. This medium was known to support growth of axenic cultures initiated from flax rust urediospores (unpublished results). Incubation was in the dark at 17°C.

RESULTS AND DISCUSSION

This technique of isolating fungal colonies from infected plant tissue depends on the use of enzymes which digest or critically weaken the host cell walls but not those of the fungus. When infected flax cotyledons were incubated in the enzyme mixture, plant protoplasts were produced but the fungus was left intact. The epidermis and vessel elements, however, were resistant to the action of the enzymes and fragments of these often remained attached to isolated colonies. The procedure can be simplified by making up the enzyme mixture in the medium used for axenic culture of the rust.

If the rust was sporulating at the time of isolation, the walls of the host cells adjacent to the fungal colonies were more difficult to digestion. The pellets of colonies isolated by procedure I were pale green because the three hour incubation left some apparently intact host cells in the center of the fungal mass. When procedure II was used many colonies were virtually free of contaminating host cells (Figs. III-1, III-2 and III-3). All of the 82 colonies plated on the agar medium grew, assumed a spherical shape and turned orange in colour. Microscopic examination confirmed that urediospore development had occurred (Fig. III-4). None of these spores were observed to have germinated. Three colonies continued to increase in size, the orange spheres becoming about four times the diameter of the originally plated colonies. White

mycelium began to develop from the base of these after about one month in culture and growth has continued after transfer to fresh medium (Fig. III-5). Small fragments of cotyledons attached to the colonies neither inhibited nor promoted fungal growth.

There is little chance that the axenic cultures of the flax rust fungus presently available originate from a single spore since several dozen spores in close association are required to initiate axenic growth (9). It has also been suggested that anastomosis of germ tubes precedes the development of colonies in culture (3). Inoculating cotyledons with single spores as described by Fleischmann et al. (4) and establishing axenic cultures from these infections using this technique would yield colonies derived from a single urediospore. Kuhl et al. (6) cultured several races of Puccinia graminis tritici from urediospores but growth was inconsistent. These authors suggested that the transition from sporeling to vegetative hyphae was the critical stage in the establishment of cultures capable of continued growth. The procedure described here may prove useful for the establishment of axenic cultures of other races of P. graminis as well as other rust fungi which have not been grown apart from their hosts because it bypasses in vitro sporeling development and the initiation of vegetative hyphae.

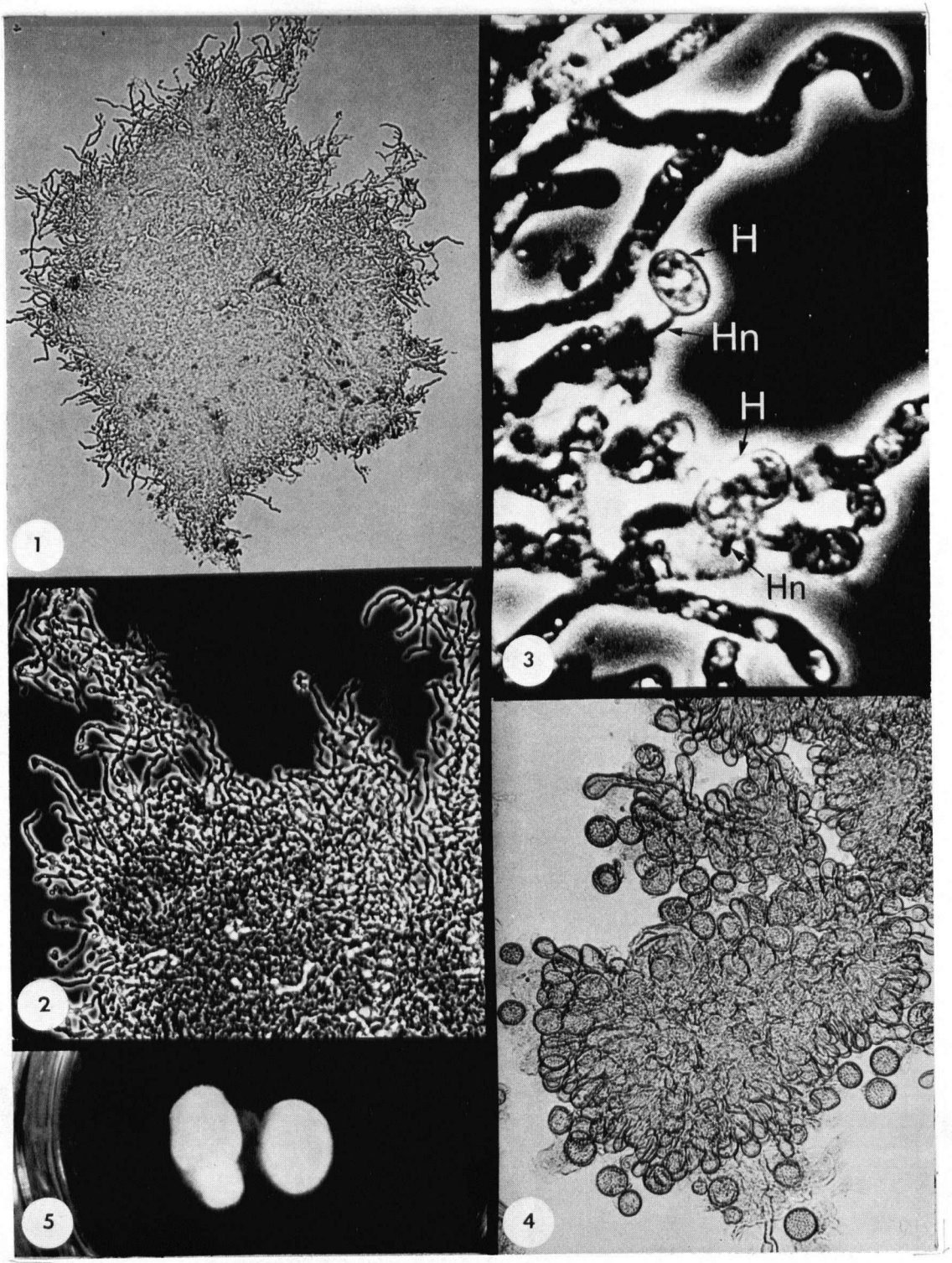
Figure III-1. A colony isolated after overnight incubation in the enzyme solution. X113.

Figure III-2. Enlargement of a colony similar to that in Figure III-1. Note that most hyphal strands have not been severed during isolation. X224.

Figure III-3. Intact haustoria (H) including haustorial neck (Hn) attached to isolated colony. X1828.

Figure III-4. Urediospores in the orange spheres which developed several weeks after the isolated colonies were plated on axenic culture medium. X224.

Figure III-5. An axenic culture which grew from an isolated colony. Photographed 2 weeks after the first transfer and about 2 months after the beginning of axenic culture. X2.5.


1
2
3
H
Hn
H
Hn
4
5

REFERENCES

1. Chen, R.F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242: 173-181.

2. Coffey, M.D., A. Bose and M. Shaw. 1970. In vitro culture of flax rust, Melampsora lini. Can. J. Bot. 48: 773-776.

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

4. Fleischmann, G., J. Khair and A. Dinoor. 1966. Dry twist: a new system of culturing rust from single spores. Can. J. Bot. 44: 1009-1013.

5. Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Expt. Cell Res. 50: 151-158.

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

7. Maheshwari, R. 1966. The physiology of penetration and infection by urediospores of rust fungi. Ph.D. Thesis, University of Wisconsin, University Microfilms, Ltd., A Xerox Co., Ann Arbor, Michigan.

8. Otsuki, Y. and I. Takebe. 1969. Isolation of intact mesophyll cells and their protoplasts from higher plants. Plant & Cell Physiol. 10: 917-921.

9. Turel, F.L.M. 1969. Saprophytic development of the flax rust Melampsora lini race no. 3. Can. J. Bot. 47: 821-823.

Chapter IV

Isolation and Axenic Culture of Poplar Rust*

INTRODUCTION

The difficulties of studying the host:parasite relations of the rust fungi are compounded because they are obligate parasites and normally cannot grow apart from their host plants. Recently, several species of rusts have been successfully grown by seeding uncontaminated urediospores onto appropriate media (1, 5). In this report a technique is described for the establishment, for the first time, of axenic colonies of poplar rust from urediopustules excised from infected leaves using a defined medium developed for flax rust (7). Like the enzymatic technique for isolation and axenic growth of flax rust which we described earlier (3) the 'excision' technique does not require uncontaminated urediospores, and is simpler.

Poplar rust (Melampsora occidentalis Pers.) is a heteroecious rust with several alternate hosts (4), in which the uredial and telial stages occur on black cottonwood (Populus trichocarpa Torr. and Gray).

---

*Published in Canadian Journal of Botany Vol. 52: 2228-2230, 1974.

MATERIALS AND METHODS

Leaves of infected cottonwood, growing on the U.B.C. campus, were collected at the beginning of October, when orange urediopustules were abundant, and sterilized by consecutive immersion in 1% sodium lauryl sulphate (20 min), 70% ethanol (2 min) and 1% Na hypochlorite (5 min), with a thorough rinse in distilled water after each treatment. Single rust pustules, including a surrounding zone of uninfected tissue 1 mm wide were excised and placed, six per petri dish (60 mm diam.), on agar medium. The dishes were sealed with parafilm and incubated at 17°C in darkness. The medium (see legend, Fig. 1) was prepared at double concentration, filtered sterilized and mixed with an equal volume of agar (purified by 5 rinses in distilled water over 24 h, 1 rinse in ethanol and 1 rinse in chloroform).

RESULTS AND DISCUSSION

Pustules on the leaf pieces doubled in size during the first week of incubation, pale stromata developing to protrude above the leaf surface. Three weeks later mycelium emerged from some pustules and after 4 months mycelium had grown onto the agar medium from 30% of the pustules. This mycelium developed from stromatic hyphae of the fungus, not from the germination of the urediospores originally present in the excised pustule. No growth of leaf tissue occurred.

Once established on the medium, colonies were roughly semi-spherical in shape (Fig. IV-1) with a compact stromatic center, in which irregularly shaped spore-like bodies were embedded (Fig. IV-2), and with a surface of fluffy white mycelium. Doubling in size occurred every three weeks during the first months of culture, but subsequently took four or five weeks. Colonies were transferred to fresh medium at three-week intervals and bisected when they reached 1.5 cm in diameter. Apparently normal urediospores were produced on the surfaces of some colonies after three months incubation (Fig. IV-4). On exposure to continuous light (2570 lux) the mycelium became orange in colour. To date, subcultures have been maintained continuously for one year, with losses occurring only when transfer was infrequent.

Reinfection of black cottonwood leaves by axenically grown poplar rust was demonstrated as follows. Newly emerged leaves 5 cm long were surface sterilized and a strip (1 cm x 1 mm) cut from the edge of one lobe. Each leaf was laid on the agar with its cut edge in contact with a rust colony. The sealed dishes were then incubated in the light (2570 lux). The fungus invaded the leaf and aerial mycelium grew from the cut surface after twenty days, then the colony which served as inoculum was removed (Fig. IV-3). Ten days later uredio-pustules developed on the leaf surfaces; urediospores collected from these pustules germinated normally.

The technique was also tested using several other species of rust. These were Hollyhock rust (Puccinia malvacearum), snapdragon rust (Puccinia antirrhini), mint rust (Puccinia mentha), thistle rust (Puccinia suaveolens), wheat rust (Puccinia graminis tritici) races ANZ-126 and 15B, and bean rust (Uromyces phaseolus) race PRE-2. Suitable pustules were processed as described above and plated onto the medium used with the poplar rust and a defined medium previously used successfully to grow wheat rust (1).

It was not possible to establish vegetative colonies of any of these rusts using the new technique. However, the wheat rust pustules (both races) grew to a limited extent when used as inoculum and plated on the medium previously used to grow wheat rust axenically using urediospores as inoculum. The growth was similar to the initial growth of flax rust (described in Chapter III). Dark brown irregularly shaped spheres grew from the inoculum increasing in size to a maximum of 0.5 cm. The spheres were composed of irregular spore-like bodies intermixed with hyphae. The growth of the wheat rust stopped about a month after the beginning of incubation.

The enzymatic (3) and excision techniques both by-pass sporeling development and the initiation of vegetative hyphae in vitro. They may therefore be useful in establishing axenic cultures of species of rust fungi which are difficult

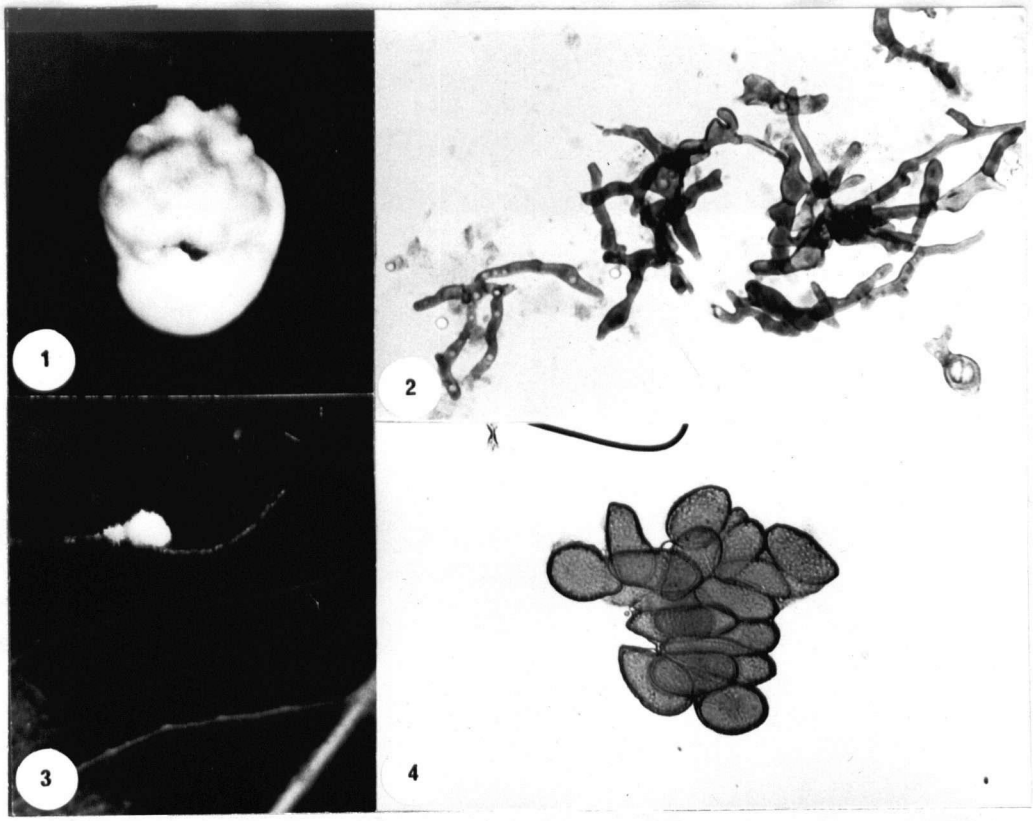
to grow from urediospores seeded directly onto the medium. Use of either technique, following single spore inoculations (2) of the host, would yield axenic cultures, and ultimately clones, each derived originally from a single urediospore. Since this cannot be accomplished by mass seeding of suitable media with urediospores, the procedure we describe should be useful in studies on the biochemical genetics of the rust fungi. The success of both the techniques described in Chapters III and IV depends on the use of a suitable medium which will support the growth of the particular species of rust under investigation.

Figure IV-1. Typical colony of Melampsora occidentalis three months after isolation. Grown on the following medium (in g/l): KNO_3, 0.25; $MgSO_4 \cdot 7H_2O$, 0.25; KH_2PO_4, 0.25; K_2HPO_4, 0.75; NH_4NO_3, 0.02; $Ca(NO_3)_2 \cdot 4H_2O$, 2.0; sucrose, 50; aspartic acid, 5.99; cysteine, 0.558; plus 0.8 ml of micronutrient stock solution containing in mg/200 ml): 13% NaFe (Geigy), 10,000; $MnSO_4 \cdot H_2O$, 447; KI, 10; $NiCl_2 \cdot 6H_2O$, 18; $CoCl_2 \cdot 6H_2O$, 18; $Ti(SO_4)_2 \cdot 9H_2O$, 42; $ZnSO_4 \cdot 7H_2O$, 35; $CuSO_4 \cdot 5H_2O$, 15; $BeSO_4$, 20; H_3PO_4 (85%), 10; H_2SO_4 (conc.), 0.2 ml.

Figure IV-2. Typical mycelium obtained from axenic cultures. Spore-like bodies were often observed. X300.

Figure IV-3. Reinfection of black cottonwood leaves. Sporulation of the pustule infecting the leaf occurred one week after this photograph was taken. X2.

Figure IV-4. Spores produced by axenic cultures of poplar rust. X500.


1
2
3
4

REFERENCES

1. Bose, A. and M. Shaw. 1974. In vitro growth of wheat and flax rust fungi on complex and chemically defined media. Can. J. Bot. 52:1118-1195

2. Fleischmann, G., J. Khair and A. Dinoor. 1966. Dry twist: a new system of culturing rust from single spores. Can. J. Bot. 44: 1009-1013.

3. Lane, W.D. and M. Shaw. 1972. Axenic culture of flax rust isolated from cotyledons by cell wall digestion. Can. J. Bot. 50: 2601-2603.

4. Molnar, A.C. and B. Sivak. 1964. Melampsora infection of pine in British Columbia. Can. J. Bot. 42: 145-158.

5. Scott, K.J. and D.J. Maclean. 1969. Culturing of rust fungi. Ann. Rev. Phytopathology 6: 123-146.

SUMMARY

1. Flax protoplasts were isolated from cotyledons. Optimum conditions of digestion, washing and manipulation were determined so that preparations consisting of 100% protoplasts with an average of 75% alive and healthy were consistently obtained.
2. A protoplast bursting bioassay procedure, based on previously published work, was developed and the conditions of its use determined so that consistent and useful results were obtained from it.
3. The theory of fungal toxin involvement in the phytopathogenic sequence involved in rust parasitism was tested using the protoplast bursting bioassay and exudates from germinating spores and pathogenic axenic cultures.
4. Evidence arguing against the fungal toxin theory was obtained and an alternate explanation, consistent with the observations which suggested this theory, was proposed.
5. Other current theories of susceptibility and resistance to obligate pathogens were discussed in relation to the evidence obtained from the protoplast bursting bioassay.
6. Factors influencing flax protoplasts maintained in culture for as long as forty days were investigated.
7. By varying these factors a diversity of types of development occurred including division of cells originating from protoplasts isolated from flax cotyledons.

8. A technique was developed for isolating intact rust colonies from parasitized leaves. The basis of this technique is the selective enzymatic digestion of host cell walls.
9. Enzymatically isolated colonies were alive after isolation and could be used as inoculum to establish axenic cultures of flax rust.
10. A new method, simpler and with several distinct advantages over the two traditionally used methods was developed, the basis of which was the use of pustules growing in parasitized leaves as inoculum.
11. Poplar rust was grown for the first time using this method.
12. The poplar rust cultures were maintained in serial subculture for a year. The colonies were shown to be pathogenic and to produce spores irregularly.