EFFECTS AND MECHANISM OF ACTION

OF Ptr (*Pyrenophora tritici-repentis*) NECROSIS TOXIN ON WHEAT

A Thesis

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Effects and Mechanism of Action of Ptr (*Pyrenophora tritici-repentis*) Necrosis Toxin on Wheat

By

Usha S Deshpande

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfilment of the requirements of the degree of

Master of Science

Dedicated to my late grandmother

and

to my parents

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ABSTRACT

Deshpande, Usha S. University of Manitoba, May 1993. Effects and Mechanism of Action of Ptr (*Pyrenophora tritici-repentis*) Necrosis Toxin on Wheat. Major Professor: Dr. G.M. Ballance.

Tan spot of wheat, caused by *Pyrenophora tritici-repentis*, is a major leaf spotting disease of wheat. Certain isolates of the fungus produce a cultivar-specific toxin, designated as Ptr necrosis toxin. The present investigation was undertaken to elucidate the mechanism and site of action of Ptr necrosis toxin. The toxin which at low concentration produces necrotic lesions on leaves of the toxin-sensitive cultivars within 48 h, had no effect on the viability of the suspension cultured cells of both toxin-insensitive, Erik, or toxin-sensitive, Glenlea, cultivars. Embryogenic calli of both cultivars, maintained on toxin-containing and toxin-free MS media, were not different from each other with respect to the growth of the calli. A response in the organ differentiation with inhibition of shoot and stimulation of root development was observed with toxin-treated Glenlea calli as a late event relative to the controls.

The effect of Ptr necrosis toxin on the loss of electrolytes, protein synthesis, chlorophyll content and accumulation of phenolic compounds was investigated at various infiltration times using the leaf tissue. The toxin-insensitive tissue was not affected at any of the post-infiltration times studied, and a steady rate of leakage was observed in both the toxin-infiltrated tissue as well as the controls. In the toxin-sensitive tissue, a differential response to the toxin in the leakage of electrolytes was observed relative to the controls. A period of 16-18 h was required for inducing permeability changes.

Protein patterns of toxin-infiltrated Glenlea leaves, labelled with ^{35}S-methionine and separated on polyacrylamide gel, produced no change in the high molecular weight proteins relative to the corresponding controls. However, the intensity of protein bands corresponding to 22 K and 14 K was lowered in the toxin-treated tissue. Chlorophyll content of toxin-infiltrated Glenlea leaves was affected only at 24 and 48 h. The toxin did not produce any effect on the level of total phenolic compounds in either cultivars. The toxin-insensitive cultivar, Erik, however, had higher phenolic levels than the toxin-sensitive cultivar, Glenlea.

1. GENERAL INTRODUCTION

Host-pathogen interaction is highly complex as it involves a series of offensive and defensive reactions between the host and pathogen. In a typical host-pathogen interaction, the pathogen utilizes numerous tactics to colonize the host plant, while the plant employs defensive strategies to avoid such an invasion. Plants possess a variety of physical and chemical defense mechanisms which are activated in response to infection. Cell wall modifications such as papilla formation and lignification, accumulation of phytoalexins and phenolic compounds, and increased peroxidase activity have been implicated to play an important role in defense mechanisms (Bell, 1981). In spite of these defensive mechanisms, the plant sometimes fails to defend itself, and in such cases, the pathogen successfully colonizes the plant causing disease.

To be successful in colonization, the pathogen may release cell wall degrading enzymes and growth hormones, or may produce metabolites or gene products which are toxic to the plant (Dimond and Waggoner, 1953). Several of these metabolites or toxins play an active role in pathogenesis. The products of the pathogen which adversely affect the plant at low physiological concentrations are designated as toxins, and are classified as host-selective (or specific) or non-host-selective (Scheffer, 1976; Rudolph, 1976).

The present investigation was undertaken to study the mechanism of toxin action of the ascomycete fungus, *Pyrenophora tritici-repentis* that causes a foliar leaf spotting disease of wheat. Commonly known as 'tan spot', this disease occurs throughout the wheat growing areas of the world (Hosford, 1982). A severe epidemic

may result in 50% yield loss (Rees et al., 1982). Although its occurrence as an endemic disease in many parts of the world was known since the 1940's, only recently has it assumed epidemic proportion with the adoption of conservation tillage practices (Hosford, 1982; Rees et al., 1982).

Susceptibility to the fungus is expressed as two distinct components of tan spot syndrome, necrosis and chlorosis (Lamari and Bernier, 1989a). Certain isolates of *P. tritici-repentis* produce a cultivar-specific toxin which is involved in the induction of tan necrosis (Lamari and Bernier, 1989c). The toxin producing ability of the pathogen was closely related to the ability of the isolate to induce tan necrosis (Lamari and Bernier, 1989c). The toxin, designated as Ptr necrosis toxin, was purified and shown to be a protein of 13,900 ± 500 molecular mass (Ballance et al., 1989).

The physiological and biochemical differences in the behaviour of the resistant and susceptible genotypes are not yet known, except that the resistant wheat cultivars are characterized by the absence of tan necrosis or chlorosis. The role of Ptr toxin in the induction of tan necrosis in susceptible wheat cultivars is well established (Lamari and Bernier, 1989c). The mechanism of action of this toxin, however, is not understood.

Effective management of many plant diseases is often based on a clear understanding of the nature of host-pathogen interactions and the factors (e.g., toxins) that are responsible for the development of such diseases. To understand such interactions, the identification of primary receptor site(s), the kinetics of toxin-target interactions together with structure of the toxin are essential. A knowledge of the site

of action of the Ptr necrosis toxin in susceptible wheat cultivars is, therefore, essential in providing a better understanding of host-pathogen interactions and resistance mechanism.

The objective of this research was to characterize the biochemical and physiological changes induced in the host plant in response to toxin exposure. The only characterized response of the toxin has been the production of necrotic lesions in the leaf tissue. Knowledge of the early detectable events which eventually lead to necrotic symptoms is essential to elucidate the potential site and mechanism of action of the toxin in the toxin-sensitive host. Approaches involving cell suspension, callus culture and *in situ* plant tissue were considered.

Infiltration of the toxin into the intact leaf had been the only approach tried when this project was started. This approach, while effective in demonstrating necrosis, has some drawbacks for studying other responses. Because the volume of the toxin being injected cannot be controlled accurately, the amount of toxin infiltrated into the tissue varies from leaf to leaf. In addition, at low concentration the toxin produces a localized necrotic effect at the contact site of the infiltrated region. Thus, toxin infiltrated into the leaf does not ensure that all cells receive uniform exposure to the toxin. The *in situ* tissue approach required that some of the disadvantages be minimized. To ensure uniform exposure of the cells to the toxin, high concentrations of the toxin were required to study the effect of the toxin in the leaf.

To overcome these problems associated with the use of leaf tissue, the effect of Ptr necrosis toxin was examined in cell suspension cultures and in callus induction.

Cell suspension cultures allow uniform and rapid exposure of cells to the toxin. However, it was recognized that unlike cells in the intact plant, cells in culture are rapidly growing, undifferentiated and non photosynthetic. Another approach using callus culture was also employed for elucidating the effect of the toxin on the induction and organ differentiation.

In order to exert its toxicity, the toxin must overcome the potential barriers, the cell wall and membrane before penetrating intracellularly. The receptor sites, therefore, could be located in the wall, membrane or cytoplasmic organelles of the toxin-sensitive tissue. Changes in membrane permeability and phenolic compounds and the effects on protein synthesis as well as chlorophyll content were evaluated in the present investigation.

2. LITERATURE REVIEW

2.0 Introduction

The present world population of about five billion and its projected growth create enormous pressures and demands for food and industrial raw materials. It is to crop plants, one of our precious few renewable resources, that we must look to meet most of these needs. Globally, about 88% of our caloric requirements and 90% of our protein is ultimately derived from plant sources - ample evidence of their importance to humankind (Borlaug, 1981). Just to keep up with current population growth, modern agricultural technology must double crop productivity by the year 2000 (Wittwer, 1980). The use of plants to meet the world's food needs is vital to our survival. Of the total world food harvest, plant products directly contribute about 82% of the gross tonnage whereas animal and marine products together contribute only 17% (FAO, 1988).

To meet the demand for food, humankind has developed a range of crop plants; but, considering the long history of domestication, the number of crop species involved is strictly limited. According to Wittwer (1980), some 24 crop species essentially stand between people and starvation. In approximate order of importance, these are: rice, wheat, corn, potato, barley, sweet potato, cassava, soybean, oats, sorghum, millet, sugarcane, sugar beet, rye, peanut, field bean, chickpea, pigeon pea, mung bean, cowpea, broad bean, yam, banana and coconut. Over 80% of the edible dry weight is derived from only 11 of these 24 crop species, two-thirds of which are cereals. Cereal grains, therefore, constitute the largest and the most important single

group of foods. Because of the high-yielding ability of their genotypes, cereals are expected to play an even more dominant role in the total world food supply in years to come.

Food losses occur throughout production, harvesting, threshing, drying, storage, processing, marketing, and distribution. World crop losses to the three major pest groups - insects, pathogens, and weeds - are currently estimated at about 35% of the global food production (Pimentel, 1981; Davidson and Lyon, 1987). Representative data on losses in world cereal crops from insects and pathogens are summarized in Table 1.

Losses to pests have been intensified by the use of the intensive crop production technology introduced with the green revolution. The new high-yielding cereal cultivars in use today are often more susceptible to pests than were their old counterparts. Before the green revolution, farmers usually selected seeds from individual plants that survived and yielded best under the native cultural conditions. These plants contained genes resistant to insects and pathogens, and competed successfully with weeds. Pimentel (1981) estimated U.S. preharvest losses of food plants to pests to be about 37% even with the use of modern pest control technology. Insects account for 13% of these losses, plant pathogens 12%, and weeds 12%.

Plant diseases are caused by many of the same classes of agents responsible for diseases of humans and animals. Fungi and bacteria are probably the most important in terms of distribution, diversity and total damage to plants, both in the field and

Table 1. Estimated Losses (%) In World Cereal Crops from Insects and Diseases[a]

Cereals	Insects	Diseases	Total
Wheat	5	9	14
Rice	27	9	36
Corn	12	9	21
Barley	4	8	12
Oats	8	9	17
Sorghum and Millets	10	9	19
Rye	2	3	5

[a]Source: McEwen (1978)

in storage.

The background literature will be reviewed under two major sections. The first section deals with the microbial toxins involved in the etiology of plant diseases, their chemical nature, and the possible target sites and the mechanisms for their toxic action. In the second part, literature on the role of *P. tritici-repentis* in tan spot of wheat , the distribution of the disease, symptoms, the morphology and life cycle of the pathogen, and the characteristics of the necrosis toxin produced by the pathogen will be reviewed.

2.1 Phytotoxins

Toxins produced by plant pathogenic microorganisms are important factors in the development of several diseases of cultivated crops. Although their presence was recognized as early as the second half of the 19th century (Graniti, 1991), it was not until the severe epidemic outbreak of the southern leaf blight of corn during the 1970-71 season that their importance in commercial agriculture was fully comprehended. The southern leaf blight caused by *Helminthosporium maydis* fungus had a greater one year impact on the U.S. farm economy than had any other plant disease or pest in all of its history (Scheffer and Briggs, 1981). Since then, a number of plant infecting fungi and bacteria have been shown to produce toxic compounds that cause severe losses in the agriculture sector of the world economy.

There is little consensus regarding the nomenclature on how toxins should be defined in plant pathology. The confusion partly stems from the fact that several microbial pathogens produce cell wall degrading enzymes and plant growth regulators

that cause damage to the structural integrity of plant cell walls or interfere with their normal metabolism. However, such metabolites are not normally considered toxins *per se*. Biochemical research during the past two decades on several microbial metabolites that are toxic to plants has greatly helped us to arrive at a consensus definition.

Phytotoxins or plant pathogenic toxins are defined as 'the products of plant pathogens that adversely affect plant functions at very low physiological concentrations, and which do not have enzyme, hormone, or nucleic acid character' (Strobel, 1982; Yoder, 1980; Graniti, 1991).

Prior to classifying the microbial metabolites as phytotoxins, it is essential to establish their role in pathogenicity, or the virulence of the causal organism. Several criteria have been proposed for classifying a metabolite as a phytotoxin (Yoder, 1980; Graniti, 1991; Rudolph, 1976; Misaghi, 1982). These are briefly summarized below.

1. Presence in, and isolation of the toxin from the diseased plants. It is essential to establish the presence of a phytotoxin in the infected plant. It is, however, not always possible to prove this since the toxin may be produced by the pathogen in extremely low amounts in the host plants. Similarly, it may be easily inactivated prior to or during its extraction from the infected tissue. Because of these reasons, this particular criterion is of limited value in establishing a toxin's role in plant pathogenicity.
2. Reproduction of a key step in disease development and its characteristic symptoms. This criterion is difficult to meet if the toxin cannot be readily

found or obtained from the infected plant tissue. In some instances, however, germinating conidia of the pathogen may contain the toxin that could be released prior to penetration in the host tissue. It is, however, quite likely that the toxin may not act alone in exerting its toxicity on the host plant. If the toxin could be isolated from the culture filtrates of the pathogen, according to this criterion, it should then be able to produce the typical disease symptoms attributed to that particular pathogen.

3. Correlation between pathogenicity and the level of toxin produced *in vitro.* A significant correlation has been established between pathogenicity and the level of toxin produced *in vitro* by the pathogen in the culture filtrates for a number of phytotoxic compounds. Yoder (1980), however, has described numerous factors that may influence the proper interpretation of this criterion as a pathogenicity factor. The toxin production by the pathogen in *in vitro* conditions is influenced by the composition of the medium, the physical environment in which the culture is being grown, and the type of isolate used. Thus the amount of toxin produced by the culture filtrate may not necessarily correlate very well with the rate of disease development. Yoder (1980) further mentions that such quantitative assessments about the pathogenicity of causal organism, although inconclusive, may still provide qualitative correlations between toxin production and pathogenicity or virulence of the organism.

4. Correlation between disease susceptibility and sensitivity to toxins. Host-selective toxins generally exhibit high toxicity only to the host of the toxin

producing pathogens. Several researchers suggest that such host selectivity, however, should be considered as a highly significant criterion for establishing the role of a toxin in plant pathogenesis (Yoder and Scheffer, 1973; Yoder, 1980; Wood, 1976; Graniti, 1991; Kohmoto and Otani, 1991). Furthermore, other metabolites with limited host selectivity may also be present in the culture filtrates of known saprophytic microorganisms.

5. Genetic analysis of the host and the pathogen. This criterion provides the most reliable and convincing evidence for the involvement of a toxin in pathogenesis (Yoder, 1980; Misaghi, 1982).

Other criteria that are sometimes used for establishing the pathogenicity of a phytotoxin include the breaking of disease resistance by toxins, the reduction in disease incidence by toxin inactivation and similar effects on disease development, *in vivo* toxin production, and sensitivity to the applied toxin by changes in environmental conditions (Rudolph, 1972, 1976; Daly and Deverall, 1983).

2.1.0 Classification

Phytotoxins are generally classified on the basis of their host selectivity or specificity and their roles in plant pathogenesis. The two main categories of phytotoxins are as follows:

2.1.0.0 Host-Selective (Host-Specific) Toxins

Host-selective toxins were first defined by Pringle and Scheffer (1964) as "metabolic product of a pathogenic microorganism which is toxic only to the host of that pathogen". Host-selective toxins must fulfil the following requirements (Goodman

et al., 1986):

a. The pathogen and the toxin must exhibit similar host-specificity. Similarly, the resistance or susceptibility of the host plant to the pathogen should parallel insensitivity or sensitivity, respectively, to the toxin.

b. The virulence of the pathogenic strains varies with their capacity to produce the toxin.

c. In susceptible host genotypes, the host-selective toxins must be capable of producing characteristic symptoms of the disease.

Genotypes that are resistant to host-selective toxins are generally not affected even at relatively higher concentrations of the toxin. Similarly, mutants of the pathogen that lack the genes for toxicity are unable to produce the toxin, and hence, are nonvirulent (Graniti, 1991). Host-selective toxins reported thus far in the plant pathology literature are summarized in Table 2 . The table also lists the current designations and the nomenclature of these toxins, the pathogens producing them, the host plants, and wherever known, their target site of action.

2.1.0.1 Non-Host-Selective Toxins

The non-host-selective toxins, in contrast, do not reproduce the patterns of resistance and susceptibility of the host to the pathogen. All the bacterial phytotoxins known so far and a majority of the fungal toxins belong to this group of phytotoxins (Graniti, 1991). The non-selective toxins, therefore, are toxic to many plants, regardless of whether or not the plants are hosts of the toxin-producing

Table 2. Host-specific toxin known to date (1990).

Pathogen (Pathotype)	Toxin (synonymous designation)	Host range	Target site
Alternaria alternata (Apple pathotype)	AM-toxin I, II & III	Apple	Chloroplast plasma membrane
A. teniussima	ATC-toxin	Pigeonpea	-
A. alternata (Tomato pathotype)	AL (or AAL)-toxin (I & II or Ta & Tb)	Tomato	Mitochondria? ACTase?
A. alternata (Strawberry pathotype)	AF-toxin I, II & III	Strawberry	Plasma membrane
A. alternata (Japanese-pear pathotype)	AK-toxin I & II	Japanese pear	Plasma membrane
A.. alternata (Rough lemon-pathotype)	ACR(L)-toxin I	Rough lemon	Mitochondrion
A. alternata (Tangerine pathotype)	ACT-toxin I & II ACTG-toxin A & B	Tangerine	Plasma membrane
A. alternata (Tobacco pathotype)	AT-toxin	Tobacco	Mitochondrion
Bipolaris sacchari	HS-toxin A, B & C	Sugarcane	Plasma membrane
Periconia circinata	PC-toxin (Peritoxin A & B)	Sorghum	Plasma membrane
Cochliobolus carbonum	HC-toxin I, II & III	Maize	Plasma membrane
C. heterostrophus race T	HMT-toxin	Maize (Tms cyto-plasm)	Mitochondrion
C. victoriae	HV-toxin	Oats	Plasma membrane

Source: Kohmoto and Otani (1991).

microorganisms. This group of toxins can therefore affect many economically important crops, and their total impact on global agriculture is quite serious.

2.2 Chemical Structures

With the exception of the Ptr necrosis toxin, which is the primary topic of this investigation and is a protein of 13,900 ± 500 molecular mass, almost all phytotoxins known to date have low molecular masses. The only known exception is the necrosis inducing peptides of less than 10,000 molecular mass produced by *Rhynchosporium secalis* (Wevelsiep et al., 1991). As a group, however, phytotoxins do not share any common structural features. They belong to such diverse classes as peptides or derivatives of amino acids, terpenoids, glycosides, phenolics, polyacetate α-pyrone derivatives, or a combination of these classes and several others (Graniti, 1991; Yoder, 1980; Kohmoto and Otani, 1991; Daly and Deverall, 1983; Misaghi, 1982).

2.3 Mechanisms of Action of Phytotoxins

Studying the mechanisms of action of phytotoxins is often considered the most challenging research area in plant pathology. These types of studies are often limited by a lack of knowledge of the molecular structure of toxins (Daly, 1981). A knowledge of the toxic action at the target site in susceptible host plants is essential for a better understanding of host-pathogen interactions and resistance mechanisms. Such studies also help us to better understand certain biochemical and physiological processes in plants. These processes include functions of membrane receptors and ion pumps, photosynthesis, genetic male sterility, transpiration and stomatal opening, and water uptake and translocation of nutrients (Graniti, 1991).

Several excellent reviews summarize our current understanding of the mechanisms of the toxic activity of different phytotoxins (Daly, 1981; Misaghi, 1982; Yoder, 1980; Kohmoto and Otani, 1991; Goodman et al., 1986). The toxic activity of phytotoxins characterized thus far is generally directed toward one of the target sites discussed below.

2.3.0 Mitochondria

The uncoupling of mitochondrial oxidation from ATP synthesis often results in high rates of respiration. The phenomenon was first observed and reported by Allen (1953) during his studies on diffusible toxins produced by biotrophic microorganisms. Since, in nearly all plant diseases, the rates of respiration often increase during the early stages of infection, mitochondrial respiration is generally the first target site investigated as a mechanism of toxicity of phytotoxins (Daly, 1976). The results of such studies, however, are often negative or inconclusive. For example, the host-selective toxin victorin (victoria blight of oats caused by *Cochliobolus victoriae*) causes a large increase in respiration in oats that are naturally infected by the fungus (Grimm and Wheeler, 1963). Subsequent studies by Wheeler and Hanchey (1966) did find mitochondria isolated from the infected plants to be damaged. Because mitochondria isolated from healthy plants exhibited respiratory control when treated with the toxin, these researchers concluded that the increased respiration observed was only a secondary consequence of general cellular damage.

The most well known host-selective toxin influencing mitochondrial respiration is produced by *Helminthosporium maydis*, race T, which causes southern leaf blight of

corn. Only the mitochondria obtained from the susceptible corn genotypes are affected by the T toxin. They exhibit drastic changes following treatment with partially purified T toxin. These changes include swelling of the mitochondria, disruption of the inner membranes and uncoupling of phosphorylation from oxidation (Miller and Koeppe, 1971; Gengenbach et al., 1973; Misaghi, 1982). The toxin was active on corn roots with a Texas male-sterile cytoplasm (T cytoplasm) but not on those resistant with a normal cytoplasm (N cytoplasm) (Payne et al., 1980; Gregory et al., 1980).

A secondary consequence of mitochondria being the primary target site for the action of phytotoxins is a general reduction seen in the ATP levels (Miller and Koeppe, 1971). This is due to the uncoupling of phosphorylation from the mitochondrial respiration.

2.3.1 Membrane Permeability

Changes in cell membrane permeability as a primary target site for the toxic action of phytotoxins were first reported for victorin toxin (Wheeler and Black, 1963; Black and Wheeler, 1966). These researchers observed a profound effect on the plasma membranes of susceptible oat genotypes upon treatment with the toxin.

Since these early reports, several toxins were reported to cause drastic changes in cell membrane permeability of the susceptible host genotypes. These include the *Alternaria kikuchiana* toxin (Otani et al., 1973), T toxin (Miller and Koeppe, 1971); syringomycin (Backman and De Vay, 1971), and the *Pseudomonas lachrymans* toxin (Keen and Williams, 1971).

Membrane permeability is often monitored by studying the rate of ion leakage from intact cells or by monitoring the conductivity changes and electrical potential or gradient across the cell membranes (Daly, 1981). An excessive leakage of physiologically important electrolytes such as H^+, K^+, Na^+ and Ca^{++} ions from the cells often leads to plasmolysis and cell death. In contrast, a slight imbalance in the normal physiological ratios of these electrolytes may lead to disturbances in the normal physiology and metabolism of plant cells. Since both Na^+ and K^+ are transferred across the membranes by ATP-dependent enzyme systems, the activity of these enzymes may also be affected by the action of the phytotoxin on plasma membranes (Daly, 1981).

2.3.2 Chloroplasts and Photosynthesis

Interference with chlorophyll synthesis resulting in chlorotic symptoms was first reported as a target site for the host-selective toxin, tentoxin, produced by *Alternaria alternata* (= *tenuis*) by Fulton et al. (1960). The chlorosis could be observed only when tentoxin was present in the tissue prior to or during the initiation of greening in developing seedlings. Other chlorosis-inducing toxins include tabtoxin or wildfire toxin produced by *Pseudomonas syringae* pv. *tabaci* and the T toxin (Daly, 1981). Tabtoxin is also reported to inhibit the activity of ribulose 1,5-biphosphate carboxylase (Rubisco) enzyme from tobacco plants (Crosthwaite and Sheen, 1979). Bhullar et al. (1975) and Daly and Barna (1980) observed a significant inhibition of dark CO_2 fixation and photosynthesis in the leaf discs treated with T toxin as compared to the untreated controls.

Other phytotoxins may also inhibit photosynthesis. Heichel and Turner (1972) observed an inhibition of photosynthesis and photorespiration and a doubling of dark respiration in bean leaves treated with fusicoccin, a phytotoxin produced by *Fusicoccum amygdali*. Tentoxin is also known to inhibit ATPase activity and photophosphorylation in toxin-sensitive species through its binding to chloroplast coupling factor (Steele et al., 1976, 1978).

When chloroplasts and photosynthesis are involved as primary target sites for the phytotoxins, it generally results in a significant reduction in the photosynthetic rates of plants. This in turn has a serious impact on the grain yields of the crops.

2.3.3 Enzymes and Proteins

Owens et al. (1968) are generally credited with the first report of interaction of a pathotoxin, rhizobitoxine, with β-cystathionase enzyme. Phaseolotoxin produced by *Pseudomonas phaseolicola* is known to inhibit ornithine carbamoyltransferase enzyme *in vitro* (Patil et al., 1970; Tam and Patil, 1972; Mitchell, 1979). Tabtoxin inhibits ribulose 1,5-biphosphate carboxylase enzyme involved in photosynthesis (Crosthwaite and Sheen, 1979) while tabtoxinine-β-lactam affects the activity of glutamine synthetase (Uchytil and Durbin, 1980).

An inhibition of the activity of a key enzyme or protein by the phytotoxins often results in abnormalities in the normal metabolism of the plants. A serious disturbance in a key biochemical pathway may also affect other metabolic pathways, thereby affecting the physiology of infected plants.

2.4 Tan Spot

Tan spot is a major foliar disease of wheat (*Triticum aestivum* L.). It is caused by a homothallic ascomycete, *Pyrenophora tritici-repentis* (Died.) Drechs. Its anamorph (i.e., the conidial state), *Drechslera tritici-repentis* (Died.) Shoem. is a lectotype of the genus *Drechslera* (Shoemaker, 1959, 1962; Lamari and Bernier, 1991). The fungus is also pathogenic to many grasses throughout the world, and appears to have the widest host range of any *Pyrenophora* species (Ellis and Waller, 1976; Lee and Gough, 1984; Lamari and Bernier, 1991; Shoemaker, 1962; Krupinsky, 1986).

Although the disease is known as 'tan spot' on the North American continent, in other geographical regions of the world, it is also commonly known as yellow spot, yellow leaf spot, leaf blight and yellow leaf blotch (Hosford, 1971). While there is little agreement on a universal common name for the disease, the causal agent, however, remains the same ascomycete fungus. To avoid further confusion, Rees and Platz (1992) have recently suggested to avoid the use of all other older names (e.g., *Pyrenophora trichostoma, Helminthosporium tritici-repentis, P. tritici-vulgaris*) for the pathogen.

2.4.0 Distribution

Tan spot is worldwide in its distribution. Prior to its recent emergence as a major leaf spot disease of wheat, it was endemic to many areas of the world, including India (Misra and Singh, 1972), Canada (Hagborg et al., 1972; Platt and Morrall, 1980a,b), the United States (Hosford, 1971) and Australia (Rees and Platz, 1983).

This was especially true in years when good rainfall distribution during the vegetative growth period of the wheat crop favoured the development of the disease.

The epidemiology and distribution of tan spot in Canada and the United States have been extensively studied (Tekauz, 1976; Hosford, 1969, 1971, 1976; McDonald et al., 1969, 1970; Schilder and Bergstrom, 1992; Wright and Sutton, 1990). The incidence of tan spot on Canadian Prairies was reported to be dependent upon the rainfall received during the first two months of the growing season (Tekauz, 1976). Little or no rain during this period resulted in a low disease incidence. In 1974, although infection due to *P. tritici-repentis* and other leaf spot pathogens, such as *Bipolaris sorokiniana* (spot blotch) and *Septoria avenae* f. sp. *triticea* (speckled leaf blotch) was widespread in Western Canada, the fungi caused insignificant damage to wheat. Tan spot occurred commonly in the Manitoba and Saskatchewan provinces, while its incidence in Alberta was rare. Based on his survey, Tekauz (1976) concluded that among the three leaf spot diseases of wheat, tan spot had the widest distribution and potential for foliar damage, and that it was the most important leaf spot disease of wheat in Western Canada.

Hosford (1969, 1971, 1976) has extensively studied the epidemiology of tan spot of wheat in North-Central U.S. plains. In these states, wheat is often grown over large areas under limited rotations, and wheat stubble, retained on the soil surface for conservation purposes, is often the most important source of primary inoculum. Hosford (1972) found that the primary source of inoculum in the form of ascospores was consistently present on stubble of overwintering wheat in the states of North and

South Dakota. Tan spot is now the major leaf spot disease causing reduction in yields and quality of hard red spring, winter and durum wheats in North Dakota (Hosford, 1987).

As compared to the Northern Great Plains, tan spot was not considered a serious threat in the Southern Plain states of the United States. Its incidence, however, has dramatically increased in these areas since the late 1970s. During the 1983-86 period, tan spot was the major foliar disease of wheat in Oklahoma (Hunger and Brown, 1987).

The epidemiology of tan spot is somewhat different in the northeastern as compared to the Great Plain states of the United States. For example, in New York state, most wheat fields are still under conventional tillage. Moreover, they are also isolated in space and time as a result of the hilly topography and long rotation patterns without wheat (Schilder and Bergstrom, 1992). In spite of this, tan spot is common and often severe in winter wheat fields in New York (Schilder and Bergstrom, 1989). Under these conditions, the primary inoculum is often seedborne. Schilder and Bergstrom (1992) also speculated that the seedborne nature of the tan spot pathogen may be responsible for the widespread occurrence of this disease throughout the wheat growing areas of the world.

The incidence of tan spot in Australia was first described in 1950 by Valder and Shaw (1952). It was widespread in northern New South Wales (NSW), while occurring sporadically in central NSW and southern Queensland. Although the disease appeared to be prevalent for quite some time, Valder and Shaw (1952) concluded that

its major epidemic was associated with unusually heavy rains in the 1950 crop season. Tan spot reemerged as a major leaf spot disease of wheat in Australia in the early 1970s following the introduction of stubble retention and conservation tillage practices (Rees and Platz, 1992). It is now a major uncontrolled foliar disease in the northeastern wheat belt of Australia, while it occurs sporadically in the northern part of the Western Australian wheat area and in parts of South Australia and southern NSW.

Compared to North America and Australia, the incidence of tan spot in Latin and South American countries is relatively of recent origin, despite these region's similarities in climatic conditions to other parts of the world where the disease has caused losses for a long time. It has become a major disease in the Southern Cone region of South America comprising Argentina, Brazil, Chile, Paraguay and Uruguay (Kohli et al., 1992). The prevalence and severity of tan spot started to increase in the Latin American countries only since 1982 (Gilchrist, 1992). Under Mexican conditions, the tan spot pathogen is often a part of a foliar blight complex with *Septoria nodorum* and *Septoria tritici* (Gilchrist, 1992).

The occurrence of tan spot in Europe, England and Wales was reported in the 1980s (Eschenbrenner, 1983; Cook and Yarham, 1989). In addition, tan spot of wheat was also reported from Japan (Nisikado, 1929), Israel (Kenneth, 1958), Cyprus (Parisinos, 1956), Kenya (Duff, 1954), and other countries (Conners, 1939). Thus far, the incidence of tan spot on wheat has been reported from 24 countries on all the six continents representing low to high rainfall areas under a wide range of temperatures

(Diaz de Ackermann et al., 1988).

2.4.1 Host Range

P. tritici-repentis has the widest host range of all species of *Pyrenophora* (Shoemaker, 1962). In addition to cultivated common and durum wheats and other *Triticum* species, the fungus is pathogenic to *Agropyron* species, bromegrass (*Bromus inermis*), rye (*Secale cereale*), *Poa*, and *Dactylis glomerata* (Hosford, 1971; Krupinsky, 1986; Kohli et al., 1992). It is, however, only slightly pathogenic to barley (*Hordeum vulgare*) and not pathogenic to oats (*Avena sativa*), *Elymus junceus*, corn (*Zea mays*), alfalfa (*Medicago sativa*) or flax (*Linum usitatissimum*) (Hosford, 1971).

The broad host range of the tan spot pathogen may cause serious problems in the control of the disease. Even if appropriate rotations are used, the presence of alternative hosts will probably ensure the continuous survival of the pathogen. For example, smooth bromegrass survives periods of drought and extremes in temperature. It is also adapted to a wide range of soil and moisture conditions in the Northern Great Plains as well as in Central Alberta (Krupinsky, 1982, 1986; Shoemaker and Berkenkamp, 1970). Bromegrass is also widespread along roadways and windbreaks near fields of cereal crops. Krupinsky (1986) collected 208 leaf samples from North Dakota, South Dakota, Minnesota and Montana during 1981 to 1984. Of the samples tested, 59% were infected with *Pyrenophora* spp. Krupinsky (1986) further isolated and classified the *Pyrenophora* spp. into 71 different isolates. He identified 52 isolates as *P. tritici-repentis* and 19 as *P. bromi*. He concluded that smooth bromegrass is an excellent alternative host for the tan spot pathogen.

2.4.2 Symptoms

The tan spot disease is characterized by the appearance of yellow to tan leaf lesions, which are either elliptic or diamond shaped to elongate irregular spots (Lamey and Hosford, 1982). The lesions are generally 1/8-1/2" long and 1/16-1/8" wide, and have a tiny dark brown spot near the centre. The lesions tend to have a narrow to broad yellow border. This unusual pattern of a tiny dark spot in a tan lesion and a yellow border produces an "eye spot" type of symptom (Lamey and Hosford, 1982) (Figure 1). As the disease progresses further, numerous such tiny spots coalesce and produce large irregular areas of dead tissue in the susceptible wheat cultivars. A severe epidemic following the heading stage seriously affects the photosynthetic capacity of the plant, thereby reducing both grain yields as well as test weight of kernels.

2.4.3 Pathogen

Till 1950s, the tan spot pathogen was classified under the genus *Helminthosporium* because it produced conidia of the *Helminthosporium* imperfect state when grown on V8 juice agar in a 12-h photoperiod. A similar fungus named *P. tritici-vulgaris* was also reported to produce leaf spots on wheat in eastern and central United States, Japan and Germany (Conners, 1939). The similarities in its life cycle with that of *P. tritici-repentis* and *P. trichostoma* prompted both Conners (1939) and Shoemaker (1962) to treat them as the same fungal species. Two researchers, Simmons (1952) and Wehmeyer (1954), have also reported a homothallic strain of *P. trichostoma* on wheat straw in Kansas that did not produce conidia. Based on the

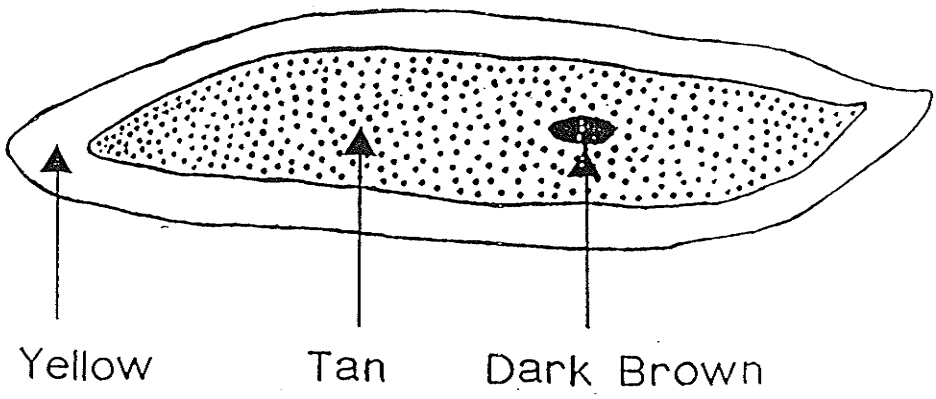


Figure 1. Schematic diagram illustrating the characteristic tan spot lesion of wheat. (Redrawn from Lamey and Hosford, 1982).

differences in sexual morphology and differing conidial states and grass hosts, Shoemaker (1959) first classified these fungi under the genus *Drechslera.* Later on, the sexual stage of this ascomycete was classified under the genus *Pyrenophora.*

A schematic representation of the life cycle of tan spot pathogen is shown in Figure 2. The pathogen is capable of propagating itself sexually by ascospores and asexually by conidia (Hosford, 1972, 1976). The fungus overwinters in wheat straw and stubble soon after harvest, and matures over the fall and winter. It produces pin-head sized sexual fruiting bodies called pseudothecia. Dark, aerial hyphae often grow on these fruiting bodies. These pseudothecia produce transparent, sac-like asci, each containing eight ascospores. A cool period is essential for the maturation of ascospores on wheat residues from the previous crop. The ascospores are forcibly released from an ascus, and are disseminated by wind. In the North American continent, this generally occurs in the beginning of June (Conners, 1939; Hosford, 1971).

The amount of primary inoculum provided by the ascospores of *P. tritici-repentis* generally has a significant effect on tan spot epidemic development in both favourable rainy and unfavourable hot and dry seasons for disease development (Adee and Pfender, 1989). The impact of the local primary inoculum level can also persist throughout an epidemic of tan spot disease, despite the important part played later by windborne secondary inoculum provided by conidia. Adee and Pfender (1989) found a high correlation between the primary inoculum levels and the disease progress during the season, as well as severity ratings near the end of cropping season.

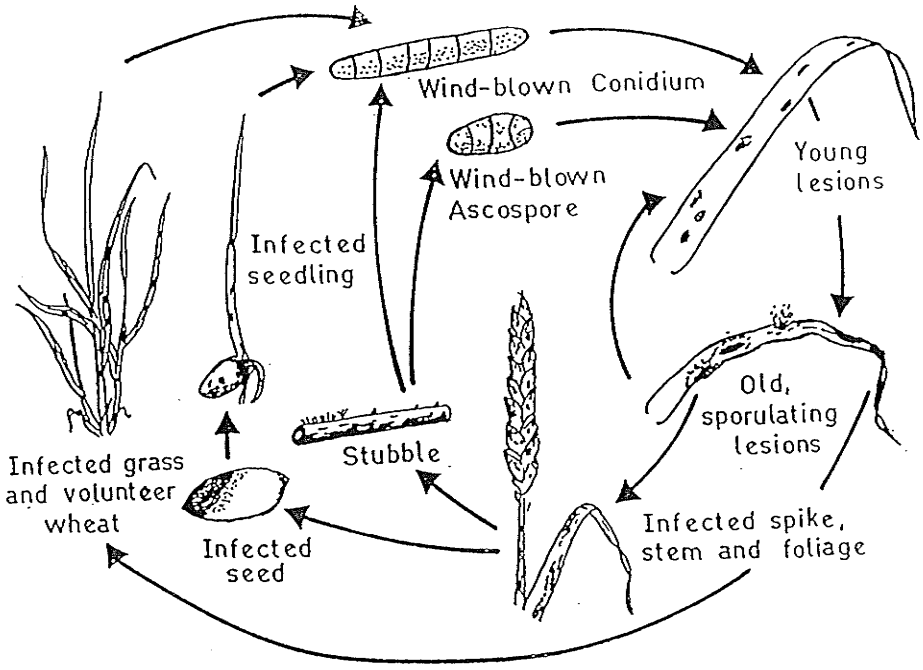


Figure 2. Schematic diagram illustrating the life cycle of the pathogen *Pyrenophora tritici-repentis*, the causal organism for tan spot of wheat (Redrawn from Schilder and Bergstorm, 1992).

In a related study, Adee et al. (1990) investigated the competitive interactions between *P. tritici-repentis* and *Septoria nodorum* using a modified plant ecology technique, commonly known as "de Wit replacement series". They inoculated wheat plants at anthesis with the two pathogens, alone and in various proportions, and analyzed the relationship between inoculum ratios and the relative yields of the crop. Adee et al. (1990) found the tan spot pathogen to be a much better competitor, since its relative yields were less affected by the presence of *S. nodorum.*

Ascospores of *P. tritici-repentis* are generally dispersed only over a short distance (Rees, 1987; Schilder and Bergstrom, 1992). Rees and Platz (1992) also observed a very strong gradient in disease level at three different sites where wheat was planted across a stubble interface. The number of lesions per leaf dropped significantly from 125 lesions/leaf in plants grown very close to the stubble interface to about 8 lesions/leaf in plants about 20 meters away from the interface. The management of crop residues in a particular field, therefore, has a large influence on the incidence and development of tan spot disease in a subsequent crop. These observations, however, may not be applicable to smaller wheat fields.

The ascospores discharged from the pseudothecia the following spring are believed to form the primary inoculum. When blown onto new green leaves of the fresh crop, they germinate under moist conditions, infect and produce the characteristic leaf spots. The number and size of leaf spots are directly related to available moisture from rainfall and dew as well as to the susceptibility of the host wheat cultivar to tan spot disease (Hosford, 1971). Severe leaf spotting, therefore, is often observed during

or following periods of wet weather and during unusually wet years.

Upon infection, the pathogen produces microscopic, dark aerial hyphae in spots on wheat leaves. As the crop develops, and if ideal moisture conditions prevail, the pathogen produces repeating cycles of asexual spores or conidia at the hyphal tips (Hosford, 1976; Wright and Sutton, 1990; Rees, 1987). The conidia are so positioned on the elevated conidiophores that they are easily dispersed by wind to neighbouring plants and fields. Conidia are primarily responsible for contributing the secondary inoculum during the crop growth season.

Several researchers have noted large numbers of conidia of *P. tritici-repentis* in the air above wheat fields on the Northern U.S. and Canadian Prairies (Hosford, 1976; Morrall and Howard, 1974; Rees and Platz, 1980). As compared to ascospores which are primarily responsible for the initial infection of the new crop, the conidia cause a rapid increase in the epidemic within a crop and spread the disease to neighbouring fields and areas. The resultant epidemic in other crops generally occurs much later, and is often less severe than in crops planted through wheat residues (Rees and Platz, 1992).

Platt and his coworkers (Platt et al., 1977; Platt and Morrall, 1980a,b) have extensively investigated the effects of substrate, temperature, photoperiod, light intensity, wind speed and humidity on the formation, growth and liberation of conidia of *P. tritici-repentis*. Although abundant mycelial growth was seen on potato dextrose agar (PDA), sucrose proline agar (SPA) and 2% malt agar, only the fungus grown on 15% V8 juice agar provided the most consistent and abundant conidiation. Autoclaved

leaves of wheat or wheatgrass [*Agropyron dasystachyum* (Hook.) Scribn.] were also found suitable as substrates for conidiation.

P. tritici-repentis also has a specific photoperiod requirement for the production of conidia. Platt et al. (1977) studied 25 different photoperiods. Although all treatments that contained a light period induced conidiation, optimum production of conidia was observed at 12 ± 1 h light per day at 21°C. In a subsequent study, Platt and Morrall (1980a) observed maximal conidiation in six isolates of *P. tritici-repentis* subjected to a 12 h photoperiod at a light intensity of 13.3 W m^{-2}. The rate of conidia production decreased significantly at lower and higher light intensities.

In a subsequent study, Platt and Morrall (1980b) exposed autoclaved wheat leaf pieces that were inoculated with the tan spot pathogen and incubated until abundant conidiation occurred on the surface, to various windspeeds and levels of relative humidity (RH) in a test chamber. The proportion of conidia liberated from conidiophores increased linearly with increasing wind speeds. Under all RH conditions tested, almost 100% liberation was observed at wind speed of 3.3 ms^{-1} and higher. However, under calm winds, increasing RH affected the conidium liberation. Dehydration of conidia and conidiophores under low RH conditions also affected their liberation. However, conidiation in *P. tritici-repentis* was not much affected at low RH as compared to that in *Cochliobolus heterostrophus* (*Helminthosporium maydis*) and *Trichometasphaeria turcica* (*Helminthosporium turcicum* Pass.) Schilder and Bergstrom (1992) subsequently confirmed these observations in an independent study.

Based on their study, Platt and Morrall (1980b) found that the conidia of *P.*

tritici-repentis were apparently more readily liberated as compared to those of other *Helminthosporia*. They also concluded that the daytime summer conditions in Western Canadian grasslands infected by *P. tritici-repentis* would almost always ensure a 100% liberation of conidia produced by the pathogen the night before. Their studies also indicate that windy and moist conditions during the crop season would greatly favour the incidence and development of a tan spot epidemic.

2.4.4 Host-Pathogen Interactions

Histopathological studies on wheat leaves sprayed with conidial suspension of *P. tritici-repentis* in growth chambers have revealed the speed with which the pathogen establishes itself in the susceptible host plants. Loughman and Deverall (1986) observed that conidial germination, appressorial formation, penetration of epidermal walls, formation of intracellular vesicles and growth of intracellular hyphae in epidermal cells occur within 12 h of inoculation of wheat plants (Figure 3). During the following 12 h, the hyphae grow slowly between the mesophyll cells. At this stage, major changes in cellular physiology take place. In susceptible varieties, the mycelia grow intracellularly at a very rapid rate. Generally, the necrotic lesions begin to appear within 48-72 h after infection. Loughman and Deverall (1986) observed that ten days after infection, large and light brown necrotic lesions with a conspicuous chlorotic margin appear on the infected leaves of susceptible cultivars. In contrast, only small and dark brown spots with inconspicuous chlorosis appear on the infected leaves of resistant cultivars.

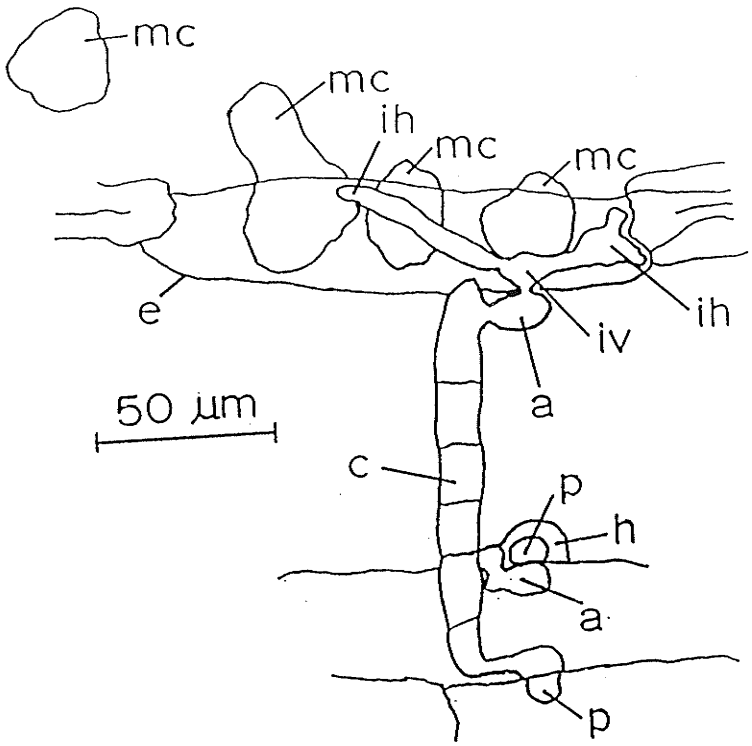


Figure 3. Infection of wheat cv. BH1146 by *Pyrenophora tritici-repentis* 6 h after inoculation showing an infected epidermal cell and two sites of attempted penetration. The abbreviations used are as follows:
a - appressorium, c - conidium, e - epidermal cell, h - halo, ih - intracellular hyphae, iv - intracellular vesicle, mc - stain retaining mesophyll cells, p - papilla.
(Redrawn from Loughman and Deverall, 1986).

Larez et al. (1986) also found that the pathogen generally enters the host epidermal cell from the appressorium with a penetration peg that subsequently develops a vesicle. Later on, the secondary hyphae that develop from the vesicle invade the mesophyll intercellularly. Larez et al. (1986) further noted that in resistant wheat varieties, the hyphae are often covered by papillae-like growth that restrict their further development. The infection in such cases is generally restricted to a few epidermal and mesophyll cells, resulting in small, brown leaf specks about 8 days after inoculation. Similar results were also reported by Aist and Israel (1977) for *Erysiphe graminis* on barley, and by Keon and Hargreaves (1983) for *P. teres.*

The early stages of *P. tritici-repentis* infection observed in wheat also appear to be quite similar to those observed with other *Pyrenophora* species attacking cereals and grasses. *P. teres* (hosts barley and oats) and *P. graminea* (host barley) also show appressorial formation above the junctions of epidermal cell walls (Keon and Hargreaves, 1983; Hargreaves, 1982). These two pathogens also showed some stomatal penetration. In contrast, Loughman and Deverall (1986) did not observe stomatal penetration by *P. tritici-repentis.* The tan spot pathogen was found to grow intercellularly through leaf mesophyll, while intracellular growth was seldom observed.

Over the years, plant pathologists have used widely differing criteria to rate disease levels. Some of these disease rating criteria include percent infection (Nagle et al., 1982), lesion size and percent infection (Luz and Hosford, 1980), number of lesions/cm^2 of leaf area (Nagle et al., 1982), an index combining lesion size, percent leaf area infected, and leaf location (Raymond et al., 1985), lesion size (Cox and

Hosford, 1987), and lesion type (Rees et al., 1988).

Recently, Lamari and Bernier (1989a) described a rating system based on lesion type to characterize host reactions to tan spot pathogen. These researchers essentially used similar lesion types described by Hosford (1971). They, however, excluded two additional criteria from their rating system, viz., lesion frequency and percent leaf area infected. The rating system of Lamari and Bernier (1989a) is summarized in Table 3.

Using this rating system, Lamari and Bernier (1989a) differentiated two characteristic symptoms. They evaluated 695 wheat accessions for reaction to a single *P. tritici-repentis* isolate in a growth chamber. Lamari and Bernier (1989a) observed both tan necrotic and chlorotic symptoms independently. About 6% of the cultivars and accessions tested in their study showed extensive chlorosis covering almost the entire leaf. These researchers also observed a high level of resistance to tan spot in di-, tetra-, hexa- and octoploid wheats; the wild wheat species generally showing a greater percentage of resistant genotypes than the cultivated higher-ploidy wheats.

In a subsequent study, Lamari and Bernier (1989b) tested 92 isolates of the tan spot pathogen from Western Canada for virulence on 11 accessions and commonly grown wheat cultivars using the rating system described above. They observed a marked difference in the ability of the different isolates to induce tan spot symptoms as manifested by tan necrosis and/or chlorosis in wheat cultivars. These symptoms appeared to be a result of specific interactions between individual isolates of the fungus and wheat genotypes.

Table 3. Ratings Criteria Based on Lesion Type to Characterize Host Plant Reaction to *P. tritici-repentis*[a]

Rating	Characteristics
1	Small dark brown to black spots without any surrounding chlorosis or tan necrosis (Resistant Type)
2	Small dark brown to black spots with very little chlorosis or tan necrosis (Moderately Resistant Type)
3	Small dark brown to black spots completely surrounded by a distinct chlorotic or tan necrotic rings; lesions generally not coalescing (Moderately Resistant to Moderately Susceptible Type)
4	Small dark brown or black spots completely surrounded with chlorotic or tan necrotic zones; some of the lesions coalescing (Moderately Susceptible)
5	The dark brown or black centres may or may not be distinguishable; most lesions consist of coalescing chlorotic or tan necrotic zones (Susceptible Type)

[a]From Lamari and Bernier (1989a)

The different isolates could be grouped broadly into three pathotypes. Pathotype I included 88% of all isolates tested from Western Canada (Lamari and Bernier, 1989b). Depending on the cultivar response, this pathotype could selectively induce either tan necrosis or extensive chlorosis, and was denoted as $nec^{+}chl^{+}$. Only two of the 92 isolates tested were grouped as Pathotype II which induced only tan necrosis but no chlorosis. They were designated as $nec^{+}chl^{-}$. In contrast, isolates classified as Pathotype III could induce only the chlorosis symptoms. These pathotypes were classified as $nec^{-}chl^{+}$. Of the 92 isolates tested, nine isolates belonged to this category.

The identification of the fourth avirulent pathotype was later reported by Lamari et al. (1991). Lamari and Bernier (1989b) further observed that the specific host reaction was expressed only when the fungus had grown intercellularly in the mesophyll. This study, moreover, also confirmed their earlier findings (Lamari and Bernier, 1989a) that the tan spot symptom consisted of two distinct symptoms, viz., tan necrosis and chlorosis.

2.5 Ptr Necrosis Toxin: Identification and Properties

The role of *P. tritici-repentis* in the etiology of tan spot disease of wheat was well known and well established during the 1970s. Researchers also had long speculated that toxins, similar to those produced by *Helminthosporium* spp., may be involved in the expression of tan necrosis and chlorosis. The most compelling reason for this was that leaf chlorosis often occurs far away from the lesions produced by the pathogen, suggesting that a mobile toxic metabolite of the pathogen may be involved.

Similarly, often times the tissues collapse suddenly and die at a much faster rate than could be expected from the mere presence of the pathogen (Rees and Platz, 1983).

Using a similar rationale of chlorosis expanding rapidly and apparently ahead of the colonized area, Tomas and Bockus (1987) also speculated the possible involvement of a toxin in the etiology of tan spot. These researchers showed that the cell-free culture filtrates of nine different isolates of the tan spot pathogen were capable of inducing typical tan spot symptoms upon infiltration of the extract into susceptible wheat genotypes.

Tomas and Bockus (1987) also compared ten wheat cultivars, ranging from highly susceptible to highly resistant types, for their reactions to pathogen inoculation and toxic culture filtrate infiltration. A high degree of correlation ($r = 0.81$ to 1.00) was observed between the sensitivity to the toxic filtrate and the susceptibility of the cultivars to the fungal pathogen. Based on their studies, Tomas and Bockus (1987) concluded that the disease resistance in some wheat cultivars was probably due to their insensitivity to the toxin, and that the toxic culture filtrates could be used in screening wheat genotypes for tan spot resistance.

Subsequent to this study, Lamari and Bernier (1989a), for the first time, identified three virulent pathotypes in a population of the tan spot pathogen from Western Canada. These pathotypes were classified based on their ability to induce both tan necrosis and extensive chlorosis ($nec^{+}chl^{+}$), extensive chlorosis only ($nec^{-}chl^{+}$) and tan necrosis only ($nec^{+}chl^{-}$) on susceptible wheat cultivars. The development of the symptoms, i.e., either tan necrosis or extensive chlorosis, varied with the cultivar.

Lamari and Bernier (1989a) also observed that the expression of tan necrosis as well as extensive chlorosis was due to specific interactions between individual host genotypes and pathotypes of the pathogen.

Based on these and other previous studies, Lamari and Bernier (1989c) also speculated the involvement of a toxin in tan spot of wheat. They suggested two compelling reasons for this. The first includes the extensive chlorosis or necrosis observed in some wheat cultivars in response to infection by certain isolates (Lamari and Bernier, 1989a,c). Second, the histological evidence that hyphae of *P. tritici-repentis* grow intercellularly without penetrating the mesophyll cells (Lamari and Bernier, 1989c; Larez et al., 1986; Loughman and Deverall, 1986). Furthermore, Lamari and Bernier (1989c) also found that only those pathotypes capable of inducing tan necrosis (i.e., nec^+) produced toxin *in vitro* in the culture filtrates.

The initial attempts of Lamari and Bernier (1989c) to isolate and purify the toxin from culture filtrates of *P. tritici-repentis,* using an approach similar to that of Smedegaard-Petersen (1977) who isolated two toxins from *P. teres*, were not successful. They resorted to dialysing the culture filtrates through a membrane of 8,000 molecular cut-off point. Both the crude and dialysed filtrates from isolates ASC1 and 86-124 contained metabolites that induced severe tan necrosis within 24-36 h of infiltration into susceptible wheat genotypes. These symptoms were consistent with those obtained with spore-inoculated wheat plants. Moreover, the membrane used for dialysis of the culture filtrates further suggested that the toxin was a large molecule of over 8,000 molecular mass.

Lamari and Bernier (1989c), in an attempt to partially characterize the toxic metabolite, autoclaved both the crude and dialysed extracts at 121°C for 20 min. As compared to the non-autoclaved controls, both samples failed to induce tan necrosis in susceptible wheat genotypes. Based on their studies, Lamari and Bernier (1989c) concluded that the toxin of *P. tritici-repentis* was cultivar specific, involved in the induction of tan necrosis and was a pathogenicity factor. They also designated it as Ptr necrosis toxin, nomenclature that is now widely accepted.

The Ptr necrosis toxin was subsequently purified to homogeneity and shown to be a protein of 13,900 ± 500 molecular mass (Ballance et al., 1989). Ballance et al. (1989) purified the Ptr toxin from the culture filtrates of a $nec^{+}chl^{-}$ isolate, 86-124, using a combination of gel permeation (Sephadex G-100) and cation-exchange (CM-cellulose) column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the major peak constituent following CM-cellulose chromatography has shown it to be a protein of molecular mass 13,900 ± 500.

Ballance et al. (1989) further studied several physicochemical properties of the Ptr necrosis toxin. It contains three tryptophan and two cysteine residues. Its molar extinction coefficient, ε_M, was found to be 20,800 $M^{-1}cm^{-1}$. Bioassays have shown this protein to be a very potent toxin, inducing necrosis upon infiltration into sensitive wheat plants within three days at a concentration of 2.4 ng/ml. At 0.240 µg/ml, the necrotic symptoms were readily visible on sensitive plants within 24 h, while no effect was observed on the resistant wheat genotypes at concentrations as high as 24 µg/ml.

Ballance et al. (1989) also found that the toxic activity of the protein was completely lost upon heating at 70°C for 1 h; heating at 40°C for 1 h had no effect on the toxin activity, while only traces of activity were retained upon heating at 55°C for 1 h or at 70°C for 30 min. The two cysteine residues also appeared to be involved in a disulfide bond, the cleavage of which with a reducing agent, dithiothreitol (DTT), resulted in a loss of toxin activity. Proteolytic cleavage with thermolysin, however, degraded only a fraction of the Ptr necrosis toxin when incubations were carried out at 30°C: higher incubation temperatures of 40°C and 50°C for 1 h completely destroyed the protein as well as its toxic activity.

Subsequent to Ballance et al. (1989) study, Tomas et al. (1990) also purified a cultivar-specific protein toxin from Pt-1c isolate of *P. tritici-repentis*. In addition to the gel permeation and cation-exchange chromatographic techniques used by Ballance et al. (1989), these researchers also used cation-exchange high-performance liquid chromatography (HPLC) to purify the toxin to homogeneity. Tomas et al. (1990) designated it as Ptr toxin, as distinct from the Ptr necrosis toxin identified by Ballance et al. (1989). The molecular mass of the Ptr toxin was found to be 14,700. Its amino acid composition, however, appeared to be quite similar to the Ptr necrosis toxin, being rich in aspartate/asparagine, serine and glycine and containing low amounts of histidine, methionine and lysine. Although no cysteine was detected even after performic acid oxidation, Tomas et al. (1990) did find a loss of toxin activity after treatment with DTT. As compared to the Ptr necrosis toxin, Ptr toxin purified by Tomas et al. (1990) was over 450 times less active. These researchers attributed such

low activity of their Ptr toxin to differences in the bioassays used as well as assay conditions such as volume infiltrated, plant growth stage at which the bioassay was conducted and cultivar susceptibility.

Additional studies on the *in vivo* production of the Ptr necrosis toxin were recently reported by Lamari and Ballance (1992). They extracted the intercellular washing fluid (IWF) from leaves infected with nec^+ isolates 48 h after inoculation. Bioassays with this fluid showed similar symptoms as observed with the purified Ptr necrosis toxin from these isolates. When both the IWF and the purified toxin were mixed independently with rabbit Ptr-necrosis toxin antiserum and infiltrated into the susceptible wheat plants, they failed to show any necrotic symptoms. These results thus confirmed the *in vivo* presence of the Ptr necrosis toxin in the IWF.

The role of Ptr necrosis toxin in inducing tan necrosis in susceptible wheat genotypes is thus well established. The mechanism of action by which it exerts its toxicity, however, is not understood. An understanding of its site of action in susceptible wheat genotypes is important from the viewpoint of both plant pathologists and breeders who wish to devise effective control measures and to breed for inherent resistance in commercial wheat cultivars.

The present investigation, therefore, was undertaken to elucidate the mechanism and site of action of Ptr necrosis toxin of *P. tritici-repentis*. The three primary objectives of this study were:

1. To determine if wheat cell suspension cell cultures could be used to study the site of action of Ptr necrosis toxin,

2. To study the effect of Ptr necrosis toxin on callus induction in wheat cells using tissue culture techniques, and
3. To determine the effects and site of action of the toxin in the toxin-sensitive wheat cultivar, Glenlea, using leaf tissue.

In the third objective, the following parameters were evaluated.

a. Electrolyte leakage as an indicator of damage to cell membranes,
b. Effect on protein synthesis,
c. Effect on chlorophyll content, and
d. Changes in phenolic compounds of toxin-infiltrated leaves.

3. MATERIALS

3.0 Plant material: Wheat (*Triticum aestivum* L.) cultivars, Glenlea and Erik were from the 1990 and 1991 harvests at the University of Manitoba. Glenlea is a Canadian-bred spring utility wheat (Evans et al., 1972) while Erik is an American semi-dwarf spring wheat. Both cultivars were grown in a mixture of soil, sand and peat (2:1:1 ratio) at the rate of 9 seeds per pot. The plants used for all the studies were grown in a growth room, under controlled environment, with a photoperiod of 18 h. The temperature was maintained at 22°C during the light period and at 17°C during the dark period. Young, fully expanded leaves at the two-leaf stage were used for all the assays.

3.1 Fungal isolate: The $nec^{+}chl^{-}$ fungal isolate, 86-124, used for the production of inoculum had been obtained from the naturally infected cultivar BH1146 grown in a "trap nursery" at Portage-la-Prairie, Manitoba (Lamari and Bernier, 1989b). The response of the susceptible and toxin-sensitive cultivar, Glenlea, to isolate 86-124 or the toxin is a tan necrotic lesion. The resistant and toxin-insensitive cultivar, Erik, produces only a resistant 'fleck' reaction of the isolate and no symptoms of any kind to the infiltrated toxin.

3.2 Chemicals: ^{14}C-methylated molecular weight markers, N,N-dimethylformamide, catechol were from Sigma Chemical Company, St Louis, Mo; low molecular weight protein markers were from Pharmacia LKB Biotechnology Inc., Piscataway, NJ; Scintiverse scintillation cocktail was from Fisher Scientific; and ^{35}S-methionine *trans* label was from ICN Biomedical Inc., Irvine, CA. All other chemicals used were of

reagent grade.

3.3 V8-PDA medium (Lamari and Bernier, 1989a): This medium consisted of 150 ml of V8 juice (product of Campbell soups), 10 g PDA (potato dextrose agar), 3 g $CaCO_3$ and 10 g Difco bacto-agar. The medium was made to 1 litre with distilled water and autoclaved for 20 minutes.

3.4 Trace element stock: The trace element stock contained 167 mg $LiCl_3$, 107 mg $CuCl_2.H_2O$, 34 mg H_2MoO_4, 72 mg $MnCl_2.4H_2O$ and 80 mg $CoCl_2.4H_2O$. The stock was made to 1 litre with distilled water and autoclaved for 20 minutes.

3.5 Fries medium (Dhingra and Sinclair, 1985): Each litre of medium contained 5 g ammonium tartrate, 1 g NH_4NO_3, 0.5 g $MgSO_4.7H_2O$, 1.3 g KH_2PO_4, 2.6 g K_2HPO_4, 30 g sucrose 1.0 g yeast extract and 2.0 ml trace element stock. For the production of culture filtrates, 150 ml aliquots of the medium in Roux bottles were autoclaved for 20 min.

3.6 MS (10X) inorganic stock (Murashige and Skoog, 1962). Each litre of this medium contained 16.5 g NH_4NO_3, 19.0 g KNO_3, 3.7 g $MgSO_4.7H_2O$, 1.7 g KH_2PO_4, 4.4 g $CaCl_2.2H_2O$, 62.0 mg boric acid, 223 mg $MnSO_4.4H_2O$, 86 mg $ZnSO_4.7H_2O$, 8.3 mg KI, 2.5 mg Na_2MoO_4, 0.25 mg $CuSO_4.5H_2O$, 0.25 mg $CoCl_2.6H_2O$, 372 mg ethylene diamine tetracetic acid sodium salt (EDTA.Na), 278 mg $FeSO_4.7H_2O$, 1 g myo-inositol and 200 g sucrose. The stock medium was dispensed into 100 ml aliquots and stored at -20^{0}C.

3.7 B5 vitamin stock: This solution contained 1 mg/ml pyridoxine HCl, 1 mg/ml nicotinic acid, and 5 mg/ml thiamine. The stock was made to 100 ml with distilled

water and stored at 4°C.

3.8 2,4-D stock: 200 mg 2,4-dichlorophenoxy acetic acid was first dissolved in a few drops of dimethylsulphoxide and the volume made up to 1 litre with deionised water and stored at 4°C.

3.9 Standard MS medium: To prepare the liquid MS medium, 100 ml of the MS inorganic stock solution plus 5 ml of 2,4-D stock and 1 ml B5 vitamin solution were added and diluted to 950 ml with deionized distilled water. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and the solution diluted to 1 litre. About 50 ml of the medium was dispensed into each 125 ml Erlenmeyer flasks and autoclaved for 20 min. The method for solid MS medium was essentially the same as described above except that the medium was dispensed into Erlenmeyer flasks and 0.8% (w/v) Difco bacto-agar was added prior to autoclaving. Partially purified filter sterilized Ptr necrosis toxin was added to the medium to a final concentration of 21 µg/ml when the temperature of the autoclaved medium reached 40-45°C (the toxin loses its activity at 55°C). Control medium received no toxin. Approximately 25 ml of the medium were dispensed per petri plate.

3.10 SDS-PAGE solutions: Solutions were prepared according to Laemmli (1970) with the electrode buffer based on Fling and Gregerson (1986) method..

Separating gel buffer stock: 1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS (sodium dodecyl sulphate).

Stacking gel buffer stock: 0.5 M Tris-HCl pH 6.8, 0.4% SDS.

Acrylamide stock (37:1 ratio): 50% (w/v) acrylamide, 1.35% (w/v) N,N'

methylene bis-acrylamide.

SDS-PAGE sample buffer: 0.1 M Tris-HCI pH 6.8, 30% (v/v) glycerol, 1% SDS, 0.01% bromophenol blue.

Electrode buffer 10X stock: 0.5 M Tris-HCl pH 8.5, 1.9 M glycine, 1% SDS.

Ammonium persulphate: 3.3% and 10% $(NH_4)S_2O_8$.

Stain solution: 50% (v/v) methanol, 10% glacial acetic acid, 0.025% Coomassie brilliant blue.

Destain solution: 20% (v/v) methanol, 8% glacial acetic acid in water.

3.11 Reagents for quantitation of phenolic compounds using Prussian blue assay:

Ferric chloride solution: 0.1 M $FeCl_3$ in 0.1 N HC1.

Ferricyanide reagent: 0.008 M $K_3Fe(CN)_6$. The solution was prepared fresh prior to use.

4. METHODS

4.0 Production of culture filtrates

The inoculum production was according to the method of Lamari and Bernier (1989a). One cm pieces of leaf samples of the susceptible cultivar, Glenlea, infected with Ptr isolate 86-124 were placed on a wet filter paper in petri plates to maintain high humidity. Conidiophore production was initiated by placing the petri plates under fluorescent light for 24 h at room temperature (20-25°C), followed by incubation in the dark at 20°C for a period of 24 h. Single conidia were transferred to V8-PDA medium and incubated at 20°C in the dark until the colony reached 3-4 cm in diameter. Small plugs, 0.5 cm in diameter, were cut using a sterile cork borer. The conidial plugs, thus harvested, were stored under sterile conditions at -70°C until used to produce inoculate cultures.

The method of Lamari and Bernier (1989a) was used for the production of culture filtrates. Mycelial growth was initiated by transferring single conidial plugs onto petri plates containing 25 ml of V8-PDA medium. The plates were incubated in the dark at 20°C for 4-5 days till the colonies were approximately 2-3 cm in diameter. At this stage, the cultures were flooded with sterile distilled water, the mycelia flattened with the bottom of a flamed test tube, and the water decanted. The cultures were then subjected to alternate light (20-25°C) and dark (20°C) conditions for 24 h each to initiate the production of conidiophores and conidia, respectively.

After the light and dark cycles, 10-15 ml of sterile distilled water was added to the culture plates. The conidia were harvested by dislodging them under sterile water

from the outer edge of the colonies with the help of a sterile wire loop. The conidia concentration was estimated using a Fuchs Rosenthal haemocytometer (Hausser Scientific, Blue Bell, PA) and the conidial suspension was adjusted to 5000 spores/ml by dilution. Liquid cultures were grown in 1 litre Roux bottles containing 150 ml of Fries medium as described previously. Each Roux bottle was inoculated with 5 ml of the 5000 spores/ml conidial suspension and incubated at 20°C in the dark without agitation. After three weeks, the cultures were harvested by vacuum-filtration, first through Whatman No.1 filter paper and subsequently through a 0.45 μm millipore membrane filter. The filtrates were stored at -19°C until used.

4.1 Purification of Ptr necrosis toxin

Ptr necrosis toxin was purified according to the method of Ballance et al. (1989) with slight modifications. All steps were performed at 4°C. Prior to purification, the crude culture filtrates were bioassayed for toxin activity as described in section 4.2. The thawed culture filtrates were centrifuged at 9800 g for 20 min in a Beckman model J2-21 centrifuge. The culture filtrate (initial conductance 5000 μMhos) was diluted 10 times with sodium acetate buffer (10 mM, pH 5.0) to lower the conductivity to ensure toxin binding to the ion exchange column. The diluted culture filtrate was applied to ion exchange CM-cellulose column (11 x 2.5 cm) equilibrated with the same acetate buffer. The column was washed with approximately 400 ml of buffer to remove any unbound material. Eluate from the column was monitored continuously by absorbance at 280 nm. The bound fraction was eluted with a linear gradient of NaCl (0 to 0.3 M, 200 ml total volume) prepared in equilibration buffer

and 6 ml fractions were collected. The fractions thus obtained were bioassayed for toxin activity as described below. The toxin containing fractions were pooled, dialysed and concentrated to reduce the salt concentration in an Amicon concentrator under nitrogen pressure (20 psi) using a YM-5 Diaflo membrane.

The partially purified and concentrated toxin fraction was then reapplied to a second CM-cellulose column (5 x 0.8 cm) equilibrated with the same acetate buffer. After washing the column with 100 ml of buffer, the toxin was eluted with a linear salt gradient (0-0.3 M NaCl, 100 ml total volume) prepared in the equilibration buffer and 3 ml fractions were collected. After bioassaying, the active fractions were pooled, concentrated in the Amicon concentrator as described above, and stored at -19°C.

4.2 Bioassay for toxin activity

The toxin activity was assessed by infiltration of the test solution into leaves of the toxin-sensitive (86-124 susceptible) cultivar, Glenlea, and toxin-insensitive (86-124 resistant) cultivar, Erik, at the 2-4 leaf stage. Ten µl of various dilutions (1:10, 1:100, 1:1000 and 1:10,000) of the toxin in sodium acetate buffer (10 mM, pH 5.0) were infiltrated into leaves of Glenlea and Erik using a 'Hagborg' device (Hagborg, 1970). Appropriate controls using only distilled water or buffer were run in parallel to the test samples. The infiltrated leaves were examined daily for three days, and classified as (+) if necrosis was present and as (-) if the symptom failed to appear.

4.3 SDS -polyacrylamide gel electrophoresis

The purity of the stock Ptr necrosis toxin was assessed by SDS-PAGE. Polyacrylamide gel electrophoresis was conducted at pH 8.5 using the method of Fling

and Gregerson (1986), with gradient slab gels (1.5 mm thickness) of 7.5-20% monomer acrylamide concentration. The purified toxin sample (10 μg protein) and protein molecular weight markers were prepared in sample buffer (1:1 ratio) and placed in a boiling water bath for 3-4 min. After cooling, the samples were loaded into adjacent lanes and the electrophoresis run at a constant 60 V for 16 h at 9° C. The gel was fixed for 1 h in methanol-acetic acid-water, (5:1:5) and stained with Coomassie Brilliant blue solution for 12 h. The gel was then destained, first in a quick destaining solution of 50% methanol (v/v), 10% acetic acid and 40% water for 6 h, prior to complete destaining in a regular destain solution. The molecular weight marker set (Pharmacia LKB Biotechnology Inc.) contained the following proteins: A - phosphorylase b (94 K); B - bovine serum albumin (67 K); C - ovalbumin (43 K); D - carbonic anhydrase (30 K); E - soybean trypsin inhibitor (20.1 K); F - α-lactalbumin (14.4 K).

4.4 Wheat callus culture

Mature seeds of *Triticum aestivum* cultivars, Glenlea and Erik, were used. Fifty to sixty seeds were initially rinsed with distilled water to which a few drops of Tween-20 had been added. The seeds were surface sterilized by immersing in 70% ethanol (v/v) for a minute or two and rinsed three times with distilled water. After the alcohol treatment, seeds were immersed in 1% sodium hypochlorite (20% Chlorox bleach) solution to which 2 drops of Tween-20 had been added. After approximately 20 min, the Chlorox solution was decanted and the seeds were rinsed three times with sterile deionised water. The sterilized seeds were transferred to a petri plate with a

few drops of sterile water and left in the dark at room temperature (22°C) for 24 h to allow imbibition.

All operations were performed under sterile conditions in a laminar flow hood. The embryos were isolated from the imbibed seeds by peeling the pericarp and removing the endosperm with a sterile forcep. The isolated embryos were immediately plated onto sterile MS media containing '0' and '21' µg/ml filter sterilized Ptr necrosis toxin. Eight to ten embryos of each cultivar were plated in triplicate onto both the toxin-containing and toxin-free medium. The plates were maintained at 22°C in the dark and observed over a six week period.

4.5 Wheat suspension cells

Mature Erik and Glenlea embryos were isolated from the sterilized and imbibed seeds as described in section 4.4. Five to six embryos were plated onto MS media and were maintained at room temperature in the dark to initiate calli formation. After 6 weeks, each callus was chopped into small pieces and transferred to 25 ml of liquid MS medium contained in a 125 ml Erlenmeyer flask. Several such suspension culture flasks were shaken on a gyratory shaker at 125 rpm. To assess the effect of toxin on suspension cultured cells, 2 ml aliquots of cells were sampled into small petri plates and filter sterilized toxin (1.8 and 3.5 µg/ml) was added to each of the plates separately. Appropriate controls without the toxin were prepared in parallel. Cultures were maintained at room temperature on a shaker for a 48 h period. The effect of the toxin on cell viability was monitored for a period of 48 h. Cells were stained with phenosafranine and examined under a microscope. Cells which took up the stain were

assumed to be non-viable. The experiment was conducted in triplicate.

4.6 Electrolyte leakage

The toxin-insensitive and toxin-sensitive cultivars were used for the leakage studies. Glenlea and Erik leaves were infiltrated with three different dilutions (10^{-2}, 10^{-3} and 10^{-4}) of the stock Ptr necrosis toxin (0.52 mg/ml protein concentration) in acetate buffer (10 mM, pH 5.0) using a Hagborg device. These dilutions, therefore, corresponded, to 5.2 µg/ml, 0.52 µg/ml and 0.052 µg/ml of the toxin, respectively. At least 20 plants of each cultivar were infiltrated for each of the three toxin concentrations and the infiltration area was marked using a pen. Control leaves were infiltrated with sodium acetate buffer only. Plants were returned to growth room until analyzed. At 0, 2, 4, 6, 12, 16 and 18 h post-infiltration time periods, the immediate infiltrated regions (1 cm above and 1 cm below the contact site of infiltration) of the leaves were harvested after washing three times with distilled water and assayed for electrolyte leakage. Assays for each post-infiltration time period were performed independently.

To ensure a random sample of leaf pieces, the infiltrated regions were bisected along the central vein and then cut into 1 cm pieces. The cut tissues were kept on filter paper maintained on ice. Random samples of 65 pieces were then weighed and placed in screw-capped test tubes containing 10 ml distilled water. The tubes were gently rotated on a rotator shaker for 15 min. Electrical conductivity was measured with a pipette-type electrode coupled with a Markson electromark conductivity meter in each leaching solution immediately after separation from leaf tissue. The leaching

solution was then removed and replaced with fresh distilled water and returned to the rotator. The process was repeated 4 times.

At each post-infiltration time, 4 test and 4 control samples were run in parallel for the two cultivars under study. The conductivity values at 15 min and the additive values thereafter up to 60 min period were then corrected for equal tissue number, and expressed as μMhos as a mean of quadruplicate samples.

4.7 Protein synthesis

Labelling was performed according to the method of Krishnan et al. (1989) by incubating the leaf segments in the radioactive label for appropriate time period. Total protein was extracted and incorporation of the label was estimated as TCA precipitable counts using filter paper disk method (Mans and Novelli, 1961).

In this series of experiments, only the susceptible cultivar, Glenlea, was used. The seeds were germinated on moist filter papers in petri plates at 22°C in the dark, transferred to pots and grown as described in the plant material. The first fully expanded leaves at the two-leaf growth stage were infiltrated with sodium acetate buffer (control) and toxin (5.2 μg/ml), respectively, using a Hagborg device. The buffer-infiltrated and toxin-infiltrated regions at the contact site were harvested at 6, 12, 18 and 24 h post-filtration time periods. After washing three times with distilled water and excess moisture removed, the leaves were cut transversely into 2 mm long pieces. Randomly sampled pieces weighing approximately 25 mg (4 pieces) were placed in 5 ml test tubes on ice.

To the 25 mg leaf tissue, 250 µl of 20 mM Tris-HCl buffer, pH 7.5; 50 µg/ml chloramphenicol and 50 µCi of [^{35}S]- methionine-*trans* label was added. Control and toxin-infiltrated tissues were treated in the same manner. The tissue was vacuum infiltrated in a desiccator for about 6 min, and then incubated for one hour on a shaker at 24°C. At the end of the incubation period, the labelled tissue was rinsed three times with ice-cold 1 mM non-radioactive methionine, transferred to Eppendorf microfuge tubes, frozen under liquid nitrogen, and stored at -70°C for protein extraction the following day. All radiolabelling experiments were performed in triplicate.

4.7.0 Protein extraction

Total protein was extracted according to the method of Neechi et al. (1987). The leaf tissue was homogenized in 300 µl of ice cold protein extraction buffer (5% SDS, 5% 2-mercaptoethanol in 200 mM Tris-HCl, pH 7.5) in a homogenizer and the samples were stored for 12 h at -20°C. After centrifuging the extract at 12000 g, incorporation of the [^{35}S]methionine label into proteins was estimated as TCA-precipitable counts using the filter paper disk method of Mans and Novelli (1961). Triplicate aliquots (10 µl) of the supernatant were spotted on 1 cm^2 of 3 MM Whatman filter papers, air dried, boiled for 10 min in 10% TCA solution, cooled, washed twice with ice cold acetone and air dried. The air dried filter papers were then immersed in 5 ml of scintillation cocktail, and counted in a Beckman model LS-1701 scintillation counter.

4.7.1 Analysis of radiolabelled proteins

Qualitative analysis of labelled proteins was carried out by SDS-PAGE

(Laemmli, 1970) at pH 8.5 in a 12.5% polyacrylamide gel using the buffer system of Fling and Gregerson (1986). Samples of the extract supernatant from labelled tissue and ^{14}C-methylated molecular weight markers were prepared in SDS-PAGE sample buffer and placed in a boiling water bath for 3-4 min. After cooling, the samples were electrophoresed at constant voltage (60 V) for 17 h at 9°C in a 0.75 mm thick gel.

The ^{14}C-methylated molecular weight markers consisted of:
A - albumin, bovine serum (66 K); B - albumin, chicken serum (45 K); C - glyceraldehyde-3-phosphate dehydrogenase (36 K); D - carbonic anhydrase (29 K); E - P-casein (23.6 K); F - trypsin inhibitor (20.1 K); G - α-lactalbumin (14.2 K).

4.7.2 Fluorography

The gel was prepared for fluorography as described by Chamberlain (1979). The gel was fixed in gel fix solution (45% methanol (v/v), 15% TCA) for one hour, washed twice with 50% methanol and left in salicylate fluor solution [1 M sodium salicylate, 2% glycerol (v/v) and 20% methanol (v/v)] for 20 min. It was then transferred onto Whatman 3 MM filter paper, dried on a gel dryer under heat and vacuum, and subsequently exposed to X-ray film at -80°C for 10 days.

4.8 Chlorophyll determination

Twelve day old Glenlea and Erik leaves were infiltrated with purified Ptr necrosis toxin (5 μg/ml) using a Hagborg device. The controls were infiltrated with 10 mM sodium acetate buffer pH 5.0. Only a selected area of the tissue (1 cm above and below the infiltrated area) was harvested at 6, 12, 18, 24 and 48 h post-infiltration time periods. Tissues from several plants were pooled, rinsed twice with distilled

water and excess moisture removed. Four (1 cm in length and 0.55 cm width) pieces of leaf tissue were weighed and placed in capped tubes with 5 ml of N,N-dimethylformamide. Each treatment contained three replications and all operations were performed in the dark or under minimum light conditions. The extracts were stored in the dark at 4°C for 18-24 h with periodic shaking (Moran and Porath, 1980). The optical density was read at 648 and 664 nm in 1 cm cuvette to estimate chlorophyll 'b' and chlorophyll 'a', respectively, on a Hewlett Packard model 8452 A diode array spectrophotometer. The chlorophyll content was estimated using the equation of Inskeep and Bloom (1985) and expressed as μg chlorophyll content/cm^2 of leaf area.

$$\text{Total chlorophyll content} = 17.95\ A_{647} + 8.08\ A_{664.5}$$

4.9 Estimation of phenolics using the Prussian blue assay

The Prussian blue assay (Price and Butler, 1977) is based on the reduction of ferric iron to ferrous iron by tannins and other small and large molecular weight phenolic compounds. This reductive reaction is followed by the formation of a ferricyanide-ferrous complex. The coloured product, commonly known as Prussian blue, absorbs maximally at 720 nm. One ml of the N,N-dimethylformamide extract obtained as described above was added to 25 ml of distilled water. To this 1.5 ml of ferric chloride solution in 0.1 N HCl was added, followed by the addition of 1.5 ml of 0.008 M potassium ferricyanide reagent (Price and Butler, 1977). The optical density was read at 720 nm after 10 min. The results were expressed on a per leaf area basis as μg of catechol equivalents from a standard curve prepared with catechol under the

same conditions used in the analysis. All extractions were carried out in triplicate and the individual assay run in duplicate using appropriate sample and reagent blanks. All the assays were conducted at room temperature (24°C) .

4.10 Statistical analyses

Wherever applicable, data were analyzed by the general linear model and ANOVA procedures of the Statistical Analysis System (SAS, 1985). Contrast statements were used for the comparison tests.

5. RESULTS

5.0 Toxin Purification

Culture filtrate of the $nec^{+}chl^{-}$ fungal isolate, 86-124, grown in the dark at 22°C, was the source of the toxin for purification. The culture filtrate, after dilution with equilibration buffer, was loaded onto a CMC-column, the column washed with equilibration buffer and then eluted with a linear salt gradient. A major peak of coloured material was eluted in the unbound fraction. The absorbance (280 nm) profile of the material eluted by the salt gradient is shown in Fig 4. The column effluent was bioassayed for toxin activity and fractions 42-54 which corresponded to the major protein peak were found to contain toxin activity. These fractions were pooled, dialysed, concentrated to 20 ml and applied to a second CMC-column and eluted as described above. A small amount of unbound material was washed through the second column. The toxin was eluted in the middle of a linear salt gradient (Fig 5) and coincided with the single major peak. Fractions 15-18 were pooled, dialysed with five changes of 10 mM sodium acetate buffer in an Amicon concentrator using YM-5 membrane.

Several such batches of purified toxin were pooled, concentrated and used as a stock for all the studies. The stock toxin concentration was estimated from absorbance at 280 nm using the ε_M of the toxin (Ballance et al., 1989) and found to be 0.52 mg/ml.

5.1 Assessment of Toxin Purity

The stock toxin concentrate was assessed for purity by SDS polyacrylamide gel

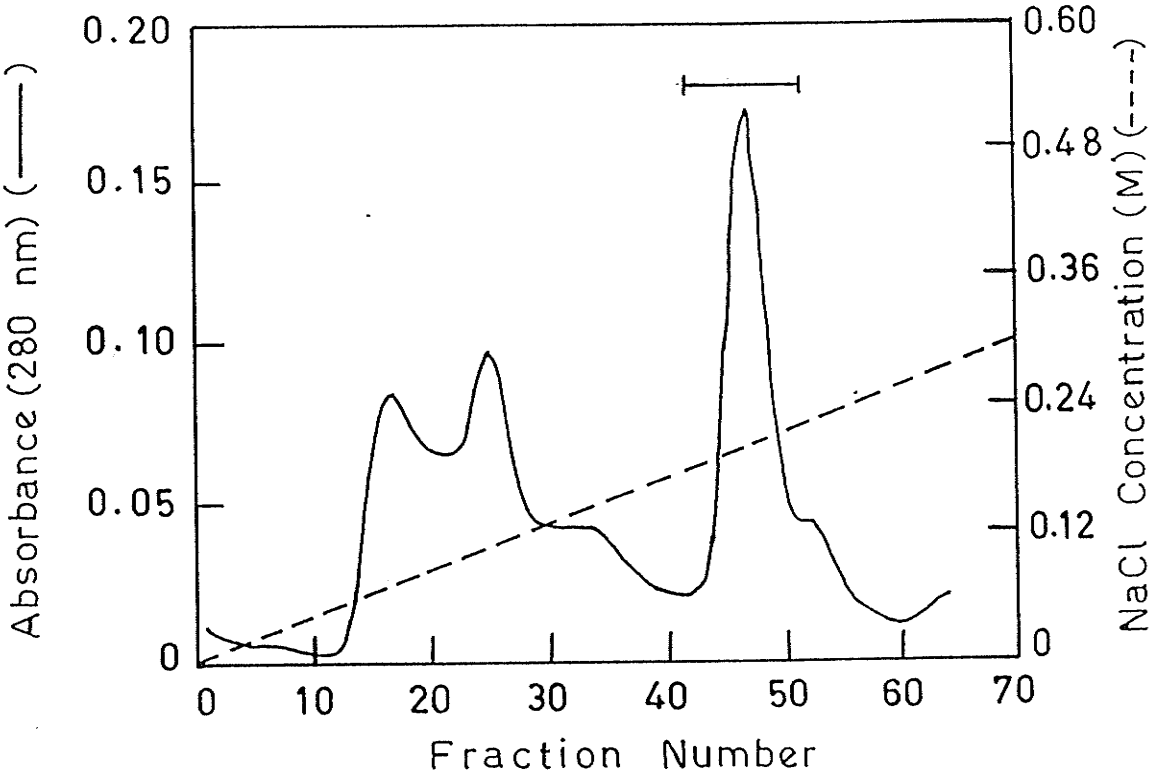


Figure 4. CM-cellulose column chromatographic profile of culture filtrate from P. tritici-repentis. The column was eluted with linear salt gradient of 0.3 M NaCl in 10 mM sodium acetate buffer, pH 5.0. The solid bar indicates fractions which contained toxin activity and were pooled and concentrated for further purification.

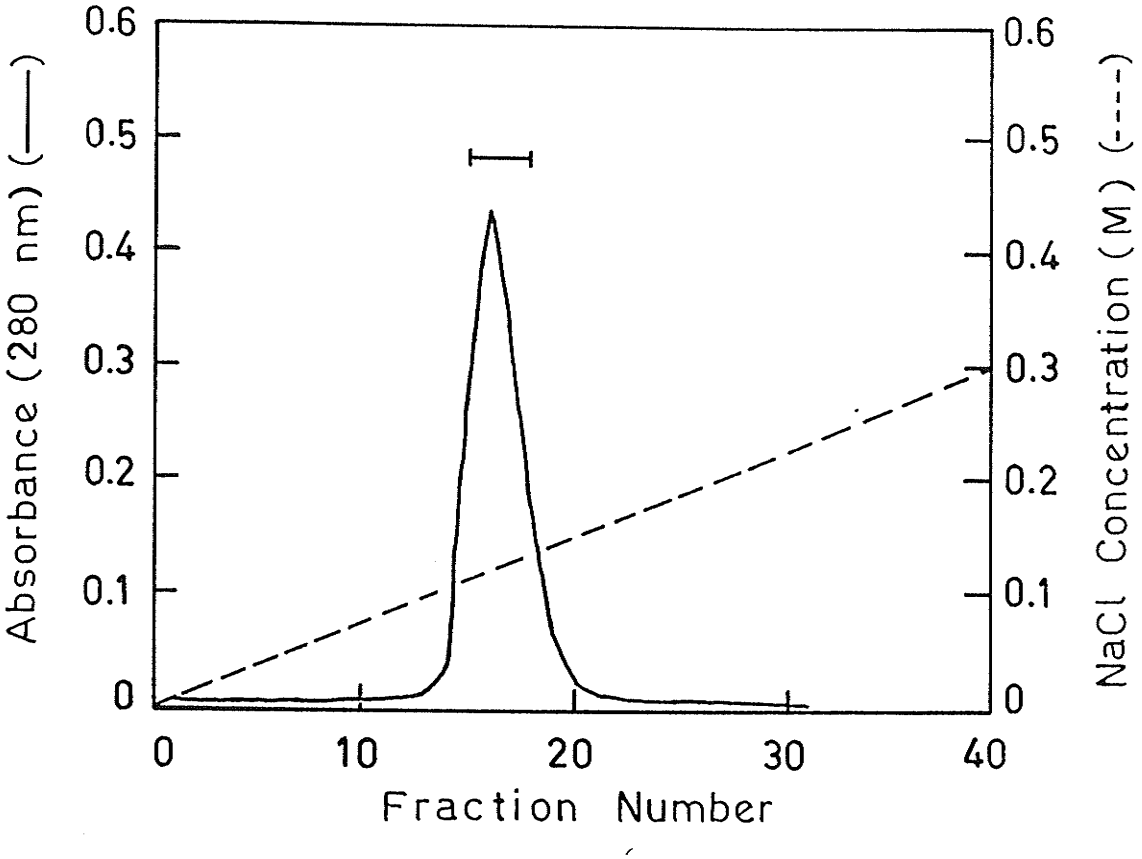


Figure 5. CM-cellulose ion exchange chromatographic profile of the pooled toxin fractions eluted from the first column. The toxin was eluted with a linear salt gradient of 0.3 M NaCl in 10 mM sodium acetate buffer, pH 5.0. Fractions 15-18 indicated by a solid bar were pooled and concentrated.

electrophoresis and protein staining. Electrophoretic analysis revealed only a single band (Fig 6), which corresponded to a protein with a molecular mass 13,900 ± 500 by comparison with standard protein molecular weight markers.

5.2 Toxin Bioassay activity

The purified stock toxin activity was bioassayed for relative activity at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions, corresponding to toxin concentrations of 52, 5.2, 0.52 and 0.052 µg/ml respectively. Leaves were examined daily, and the necrotic symptoms observed under the experimental conditions at the end of 3 days are shown in Fig 7. The resistant cultivar Erik did not show any symptoms after 3 days when tested at 10^{-1} toxin dilution. In Glenlea, at 10^{-2} and 10^{-3} dilutions, while extensive necrosis was observed by 3 days, visible necrosis could be detected as early as 18 h at the contact site. The zone of necrosis relative to the infiltrated area was reduced with a decrease in toxin concentration. At 0.052 µg/ml toxin concentration, necrosis was detected only in the immediate infiltrated region rather than the entire zone by 3 days.

5.3 Effect of Ptr necrosis toxin on wheat cell suspension cultures

To study the effect of Ptr necrosis toxin on cultured cells, suspension cell cultures were initiated from established embryogenic callus from wheat cultivars, which were sensitive and insensitive, respectively, to the toxin.

The resulting suspension cultures of the toxin-sensitive and toxin-insensitive cultivars in MS media were sampled (2 ml) and incubated at two different toxin concentrations (1.8 and 3.5 µg/ml). The effect of toxin on the cells was monitored for a period of 48 h. Cells were stained with phenosafranine and cell viability was

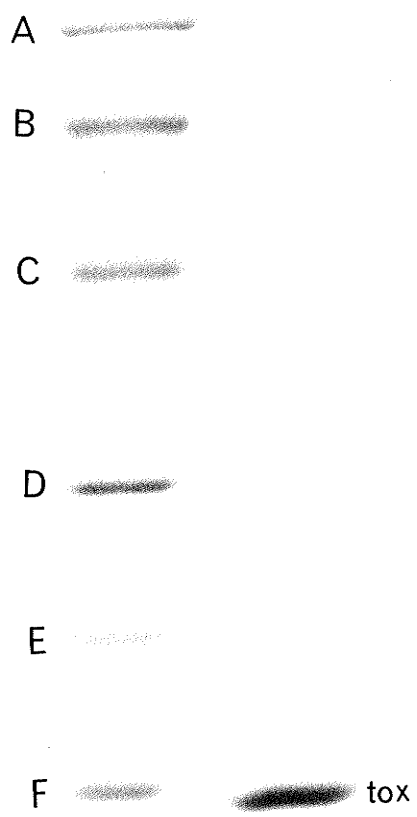


Figure 6. SDS-Polyacrylamide gel electrophoresis of purified Ptr necrosis toxin. The letters 'A-F' represent molecular weight markers (94, 67, 43, 30, 20.1, 14.4 K, respectively) as described in the Methods section.
'tox' represents 10 µg purified toxin.

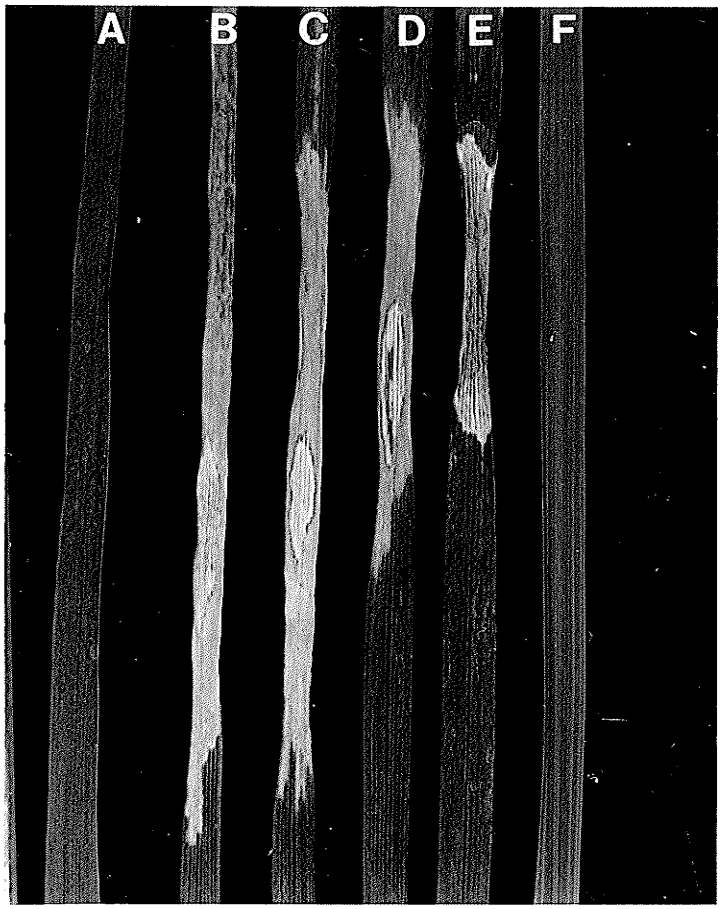


Figure 7. Necrotic symptoms induced in wheat 3 days after infiltration with various dilutions of 0.52 μg/ml purified Ptr toxin. Shown are Glenlea leaves infiltrated with sodium acetate buffer (A), 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the toxin in sodium acetate buffer (B-E), respectively. 'F' represents Erik infiltrated with 10^{-1} dilution of the toxin.

monitored periodically. Triplicate analysis of the toxin-treated and toxin-free Erik cells revealed no effect of the toxin. In both cases cells were mostly single and elongated in shape (only single cells were examined). About 95% of the toxin-free and toxin-treated Erik cells did not take up the stain, and were viable when monitored for a period of 48 h. Similarly, the toxin did not produce any effect on Glenlea cells. About 90% of the cells in both treatments, remained viable, when monitored for a period of 48 h.

5.4 Effect of Ptr necrosis toxin on wheat callus

Embryogenic calli, initiated from toxin-insensitive, Erik, and toxin-sensitive Glenlea, were maintained on toxin-containing (21 μg/ml) and toxin-free MS medium. The effect of Ptr necrosis toxin on the establishment, growth, morphology and amount of necrosis on the calli was evaluated over a 6 week period.

The embryos, excised from the resistant and susceptible cultivars, initiated callus formation on both toxin-containing and toxin-free media. Both cultivars, Erik and Glenlea, responded well in culture, with 90% of the excised embryos producing friable callus. However, Glenlea calli were much slower in growth compared to Erik calli.

The effect of Ptr toxin on callus growth and morphology is summarized in Tables 4 and 5. The growth rate of the calli from toxin-insensitive cultivar Erik was the same when grown in the presence or absence of the toxin (Fig 8). There was no difference in the morphology of the calli. The shoot and the root formation from Erik calli on toxin-containing media did not differ from those grown on toxin-free medium

Table 4. Induction of callus, organogenesis and amount of necrosis from cultured embryos of the toxin-insensitive cultivar Erik.

Experiment	[a]Toxin (µg/ml)	Embryos (per plate)	[b]Induction of callus	Number of shoots (per plate)	Number of roots	Necrosis
1	0	10	10	11	+	-
2	0	10	9	12	+	-
3	0	10	10	8	+	-
4	21	13	12	10	+	-
5	21	10	10	9	+	-
6	21	10	10	9	+	-

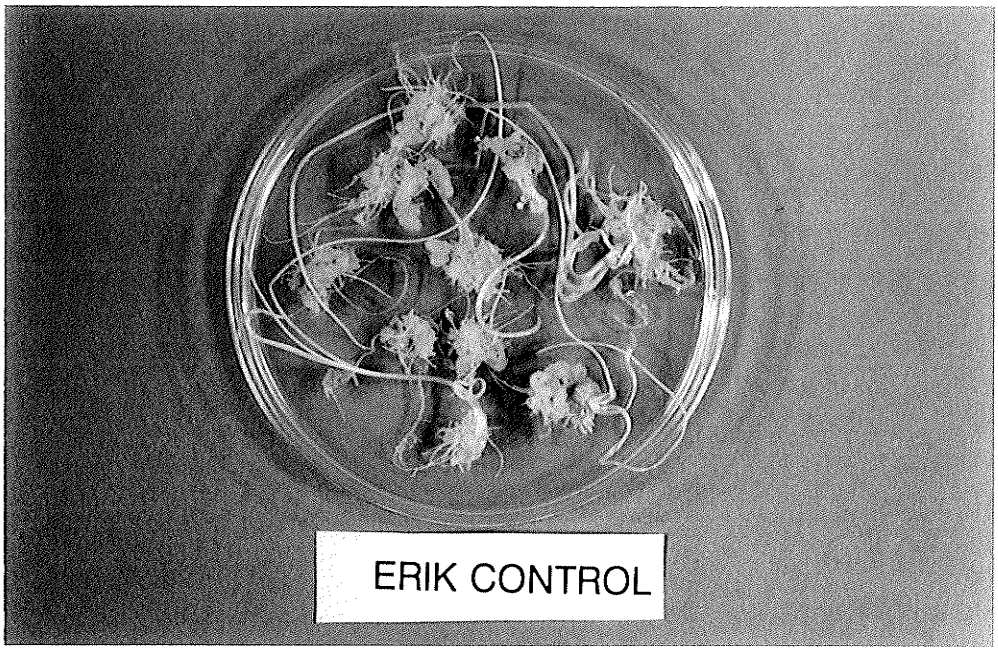
[a] Partially purified toxin used was quantified by ELISA (Engvall and Perlmann, 1979)
[b] Number of embryos per plate which had formed callus at the end of 6 week period
+Root formation was observed
- No necrosis detected

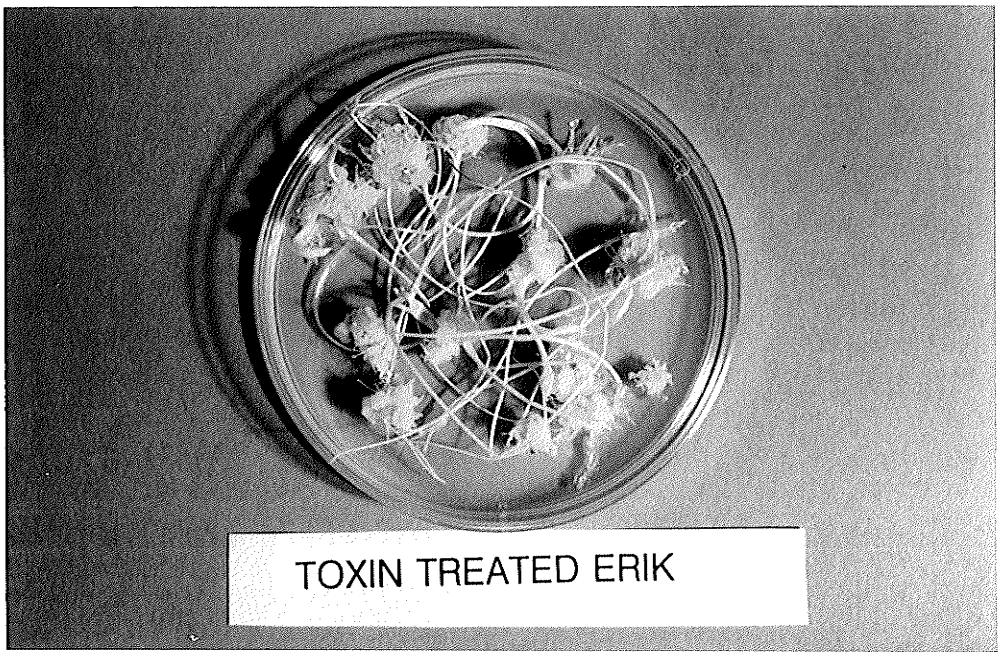
Table 5. Induction of callus, organogenesis and amount of necrosis from cultured embryos of the toxin-sensitive cultivar Glenlea.

Experiment	[a]Toxin (µg/ml)	Embryos (per plate)	[b]Induction of callus	Number of shoots (per plate)	Number of roots	Necrosis
1	0	7	7	10	+	-
2	0	7	7	9	+	-
3	0	9	9	10	+	-
4	21	9	8	3	++	-
5	21	8	8	2	++	-
6	21	9	9	5	++	-

[a] Partially purified toxin used was quantified by ELISA (Engvall and Perlmann, 1979)
[b] Number of embryos per plate which had formed callus at the end of 6 week period
+Root formation was observed
++Extensive root formation was observed
- No necrosis detected

Figure 8. Embryogenic callus of the toxin-insensitive cultivar, Erik, maintained on MS medium after 4 weeks of culture.
A - Toxin-free medium
B - Toxin-containing medium.


ERIK CONTROL


TOXIN TREATED ERIK

(Table 4). The calli formed roots as well as large (more than 1 cm long) and small shoots.

The growth rate of the toxin-sensitive calli on toxin-free media (Fig 9) did not differ from its growth on toxin-containing media. On the toxin-free media, the Glenlea calli formed roots as well as large and small shoots (Table 5). The development was similar to that of the Erik calli. The growth rate of the toxin-sensitive calli was not affected by the presence of a high toxin concentration of 21 µg/ml (Fig 9). However, a differential response with respect to organ differentiation (Fig 9) was observed at 6 weeks. The toxin-sensitive calli exposed to 21 µg/ml toxin produced minimal or no shoot development. Another change observed as a late event was a stimulation in root formation with some of the calli (Figure 9) when exposed to toxin for a longer period (8-10 weeks), in contrast to the calli maintained on toxin-free medium.

Necrosis was not detected on toxin-sensitive or insensitive-calli when exposed to 21 µg/ml toxin concentration.

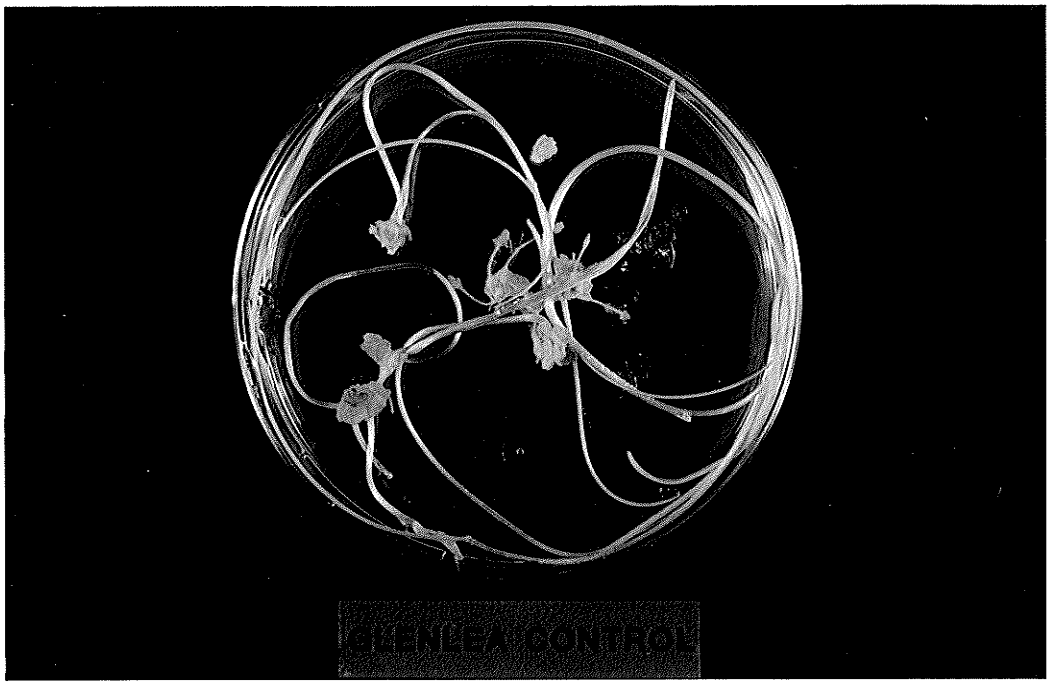
5.5 Effect of Ptr necrosis toxin on electrolyte leakage

It was observed that the fresh weight of the tissue in toxin-infiltrated Glenlea leaves declined considerably from 16 h post-infiltration times, when compared to the control treatments. Hence, conductivity was expressed on an equal sample tissue (65 leaf pieces) basis rather than weight basis.

5.5.0 Dependence on the leaching time

To evaluate the means of assessing leakage from control and toxin-treated

Figure 9. Embryogenic callus of the toxin-sensitive cultivar, Glenlea, maintained on MS medium after 4 weeks of culture.
A - Toxin-free medium
B - Toxin-containing medium.


GLENLEA CONTROL


GLENLEA TOXIN

tissues, electrolyte losses were measured over a 1 h period with replacement of the leaching solution every 15 min. Results for Erik and Glenlea are shown only for 0 and 18 h post-infiltration times, primarily to compare the rates of leakage immediately (0 h) and after sufficient exposure time (18 h) for the toxin to have had an effect. The data on the cumulative loss of electrolytes at various leaching times over a 60 min period are summarized in Figures 10A and B for a single toxin concentration (5.2 µg/ml).

A low but steady rate of leakage was observed throughout the leaching period in buffer infiltrated and toxin-infiltrated Erik (Fig 10 A). The rate of electrolyte loss from the toxin-insensitive Erik leaves, in both the 0 and 18 h toxin-treated samples, was not statistically different from the controls throughout the 60 min leaching period.

For the 0 h post-infiltration samples of buffer-infiltrated and toxin-infiltrated Glenlea, the rate of electrolyte loss was slow and steady over a 60 min leaching period (Fig 10B). In contrast, for the 18 h post-infiltration samples, the rate of electrolyte loss in toxin-infiltrated Glenlea was far greater relative to the controls. During the first 30 min period, the loss of electrolytes in the toxin-infiltrated Glenlea was almost 85% of the total loss observed during the 60 min assay period. Leakage continued during the next 30 min, but at a reduced rate in contrast to the control over the same period.

5.5.1 Dependence on Ptr toxin concentration

The toxin-insensitive cultivar, Erik, and sensitive cultivar, Glenlea, were infiltrated with 10^{-2}, 10^{-3} and 10^{-4} dilutions of a 0.52 mg/ml toxin stock which

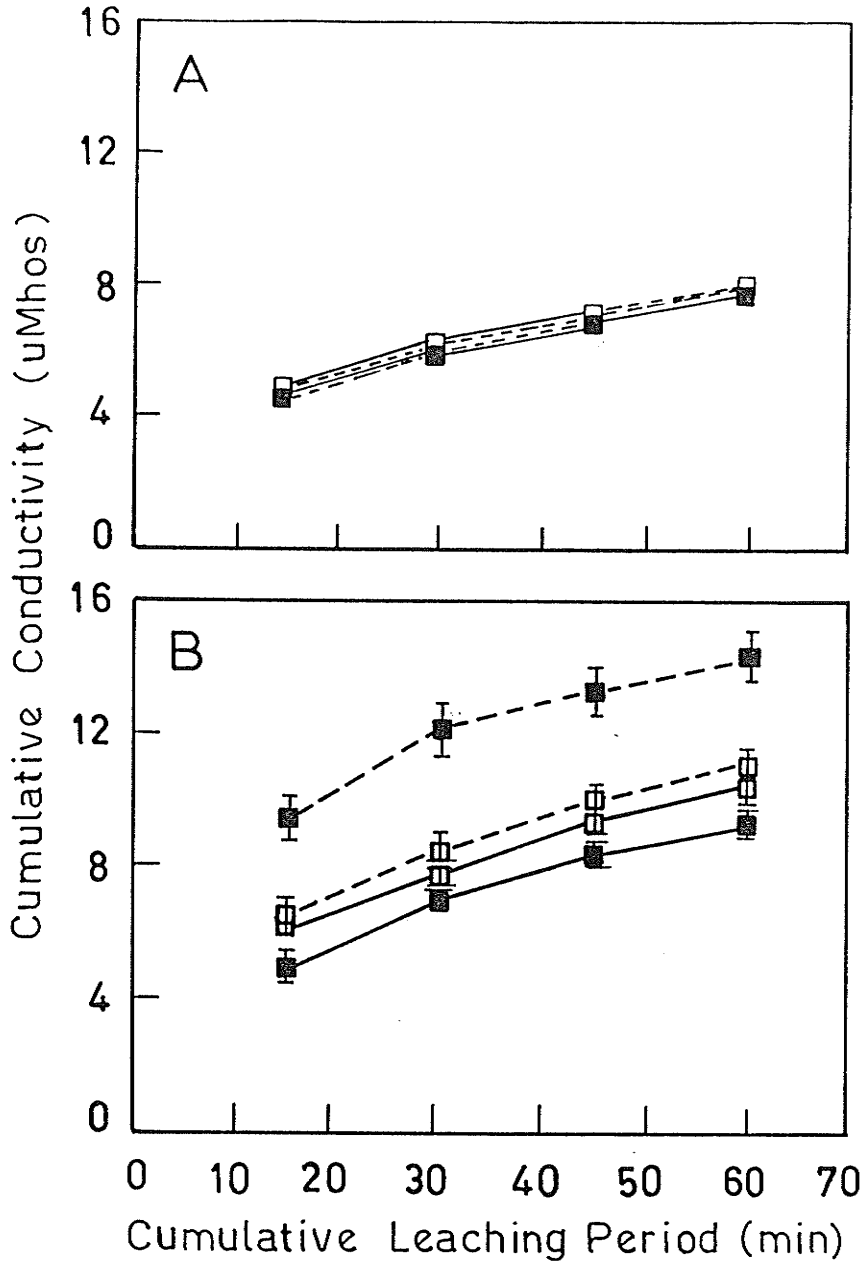


Figure 10. Effect of Ptr necrosis toxin (5.2 µg/ml) on cumulative electrolyte leakage. Cumulative leakage from 0 and 18 h post-infiltrated control and toxin-infiltrated tissues of Erik (A) and Glenlea (B) were examined. Bars represent standard error, and each point is a mean of four replicates.

0 h Control

0 h Toxin-infiltrated

18 h Control

18 h Toxin-infiltrated

corresponded to 5.2, 0.52 and 0.052 μg/ml toxin respectively. The controls were infiltrated with sodium acetate buffer. The experiment was conducted on separate days, and on different batches of plants, for all the three concentrations used in this study. Results for the three toxin concentrations with Erik and Glenlea cultivars (Fig 11) are shown only for the 18 h post-infiltration time. The cumulative conductivities monitored over a period of 1 h did not differ significantly (at 5% probability level) for Erik, with respect to the corresponding controls, even though the toxin concentration was increased 100-fold from 0.052 to 5.2 μg/ml (Fig 11). At 0.052 and 0.52 μg/ml toxin concentration, the cumulative loss of electrolytes in toxin-infiltrated Glenlea was not statistically different from the corresponding controls. However, a significant difference in the loss of electrolytes was observed for toxin-treated Glenlea leaves at the highest toxin concentration (Fig 11).

The cumulative increases in conductivity of the leaching solution for toxin-treated Glenlea samples, in general, were directly related to the toxin concentration and thus the amount of toxin infiltrated into the leaf tissue.

5.5.2 Dependence on post-infiltration time

When Erik leaves were infiltrated with sodium acetate buffer and 5.2 μg/ml toxin and the conductivity of the leaching solution measured immediately, i.e., zero-hour post-infiltration time, the conductivity was similar in both cases measuring 7.8 μMhos (Fig 12). Allowing longer post-infiltration periods (up to 18 h), prior to assessment of electrolyte leakage, still did not produce any noticeable change relative

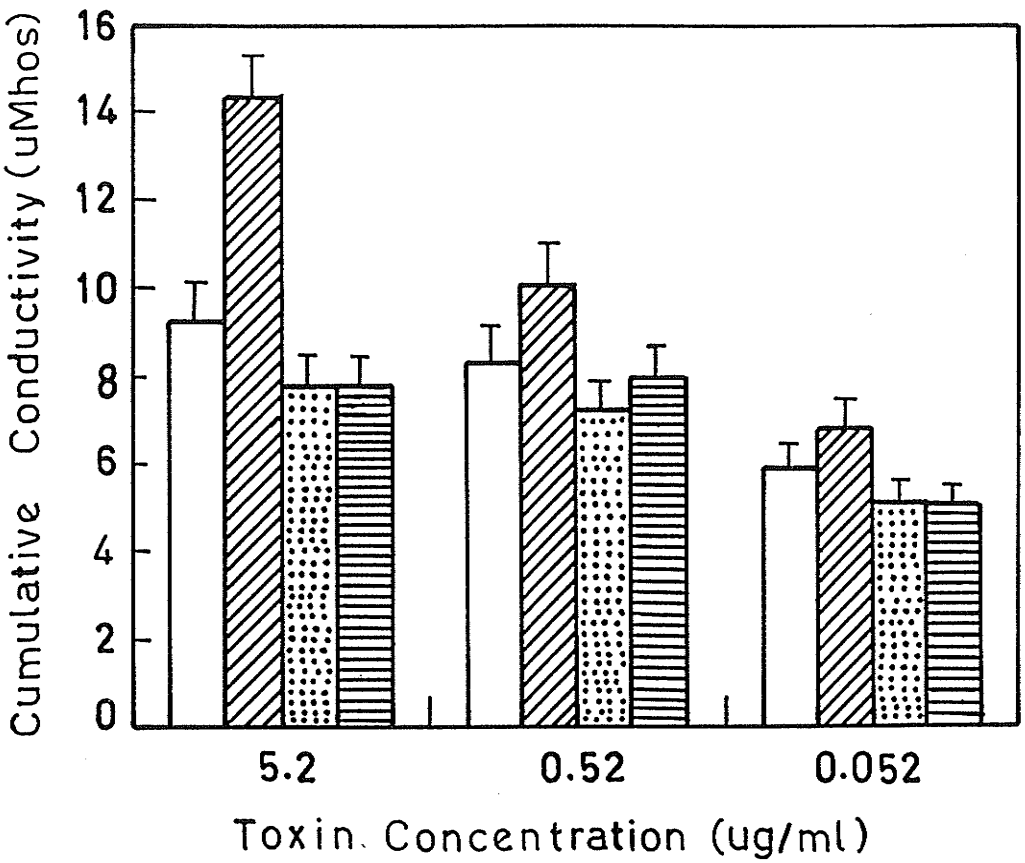


Figure 11. Effect of toxin concentration on loss of electrolytes from leaf tissue. Bars represent standard error, and each point is a mean of four replicates.

Glenlea control

Glenlea toxin-infiltrated

Erik control

Erik toxin-infiltrated

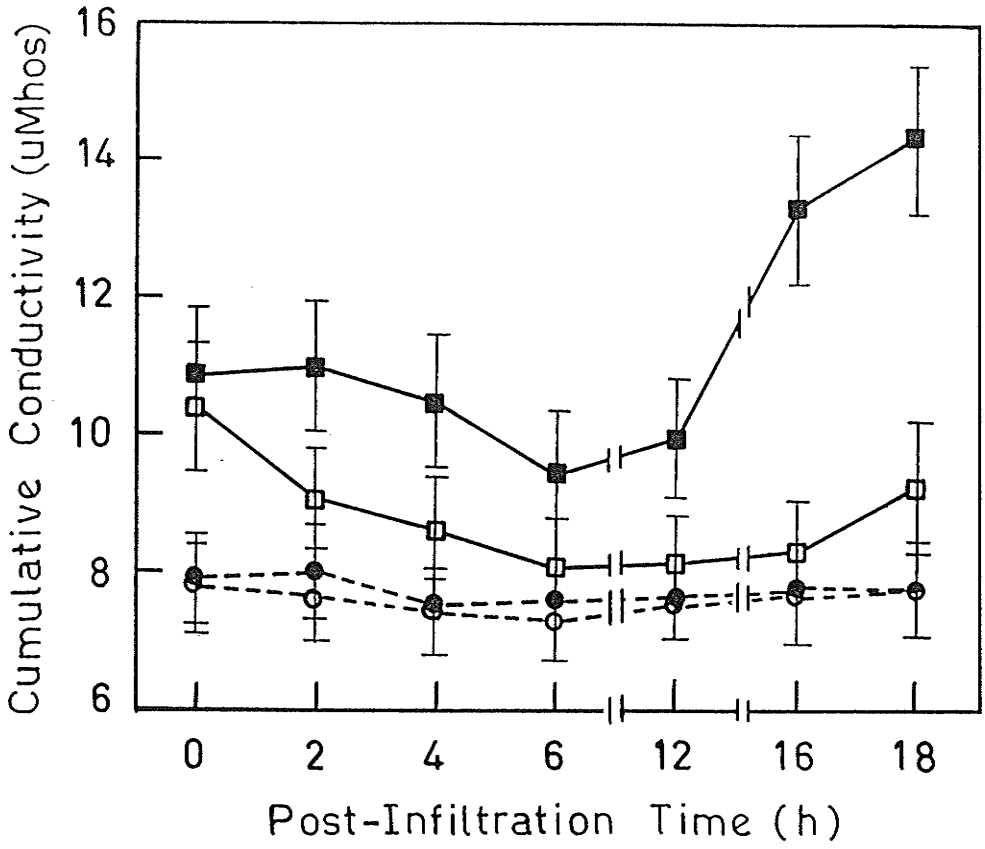


Figure 12. Electrolyte leakage at various post-infiltration times from control and toxin-infiltrated (5.2 µg/ml) leaf tissues of Erik and Glenlea. Leakage was determined by measuring changes in electrical conductivity of the leaching solution in which the tissue was suspended and shaken. Bars represent standard error, and each point is a mean of four samples.

Glenlea control

Glenlea toxin-infiltrated

Erik control

Erik toxin-infiltrated

to the controls. The cumulative conductivity values for Erik leaves varied from 7.3 to 7.7 μMhos for the control and from 7.5 to 7.9 μMhos for the toxin-infiltrated leaves (Fig 12), and were statistically not different at any of the post-infiltration times. Similarly, at lower toxin concentrations (0.52 and 0.052 μg/ml), no significant differences in the electrolyte losses were observed between the Erik control and toxin-infiltrated tissues at any of the post-infiltration time periods used in this experiment (Fig 13 and 14).

At 0 h post-infiltration time, in buffer-infiltrated Glenlea leaves, the cumulative losses in electrolytes were greater relative to those of Erik (Fig 12, 13 and 14). At longer post-infiltration times these conductivity values decreased at 6 h to levels that were comparable with those of Erik. The differences in these conductivity values may reflect the differences in the two cultivars.

When toxin was infiltrated at 5.2 μg/ml and the conductivity measured immediately, i.e., zero-hour post-infiltration time, loss of electrolytes was similar in both toxin-infiltrated and buffer-infiltrated Glenlea (Fig 12). Within the toxin-infiltrated Glenlea samples, a slight decrease in the loss of electrolytes (though not significantly different from the relative controls) was observed, when the conductivity was measured at longer post-infiltration times up to 12 h. This decrease paralleled the decrease in cumulative conductivity observed for the Glenlea control. Thereafter, a statistically significant increase in the loss of electrolytes was observed at 16 and 18 h post-infiltration time (Fig 12).

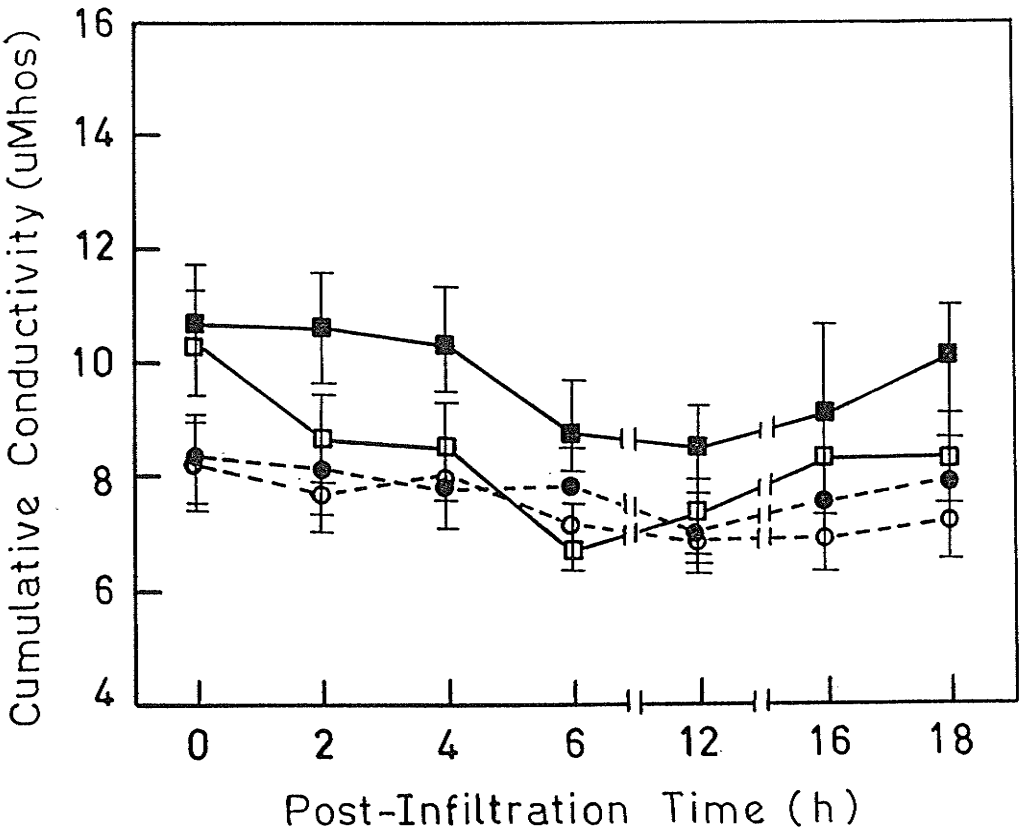


Figure 13. Electrolyte leakage at various post-infiltration times from control and toxin-infiltrated (0.52 µg/ml) leaf tissues of Erik and Glenlea. Leakage was determined by measuring changes in electrical conductivity of the leaching solution in which the tissue was suspended and shaken. Bars represent standard error, and each point is a mean of four samples

□——□ Glenlea control ■——■ Glenlea toxin-infiltrated

○----○ Erik control ●----● Erik toxin-infiltrated

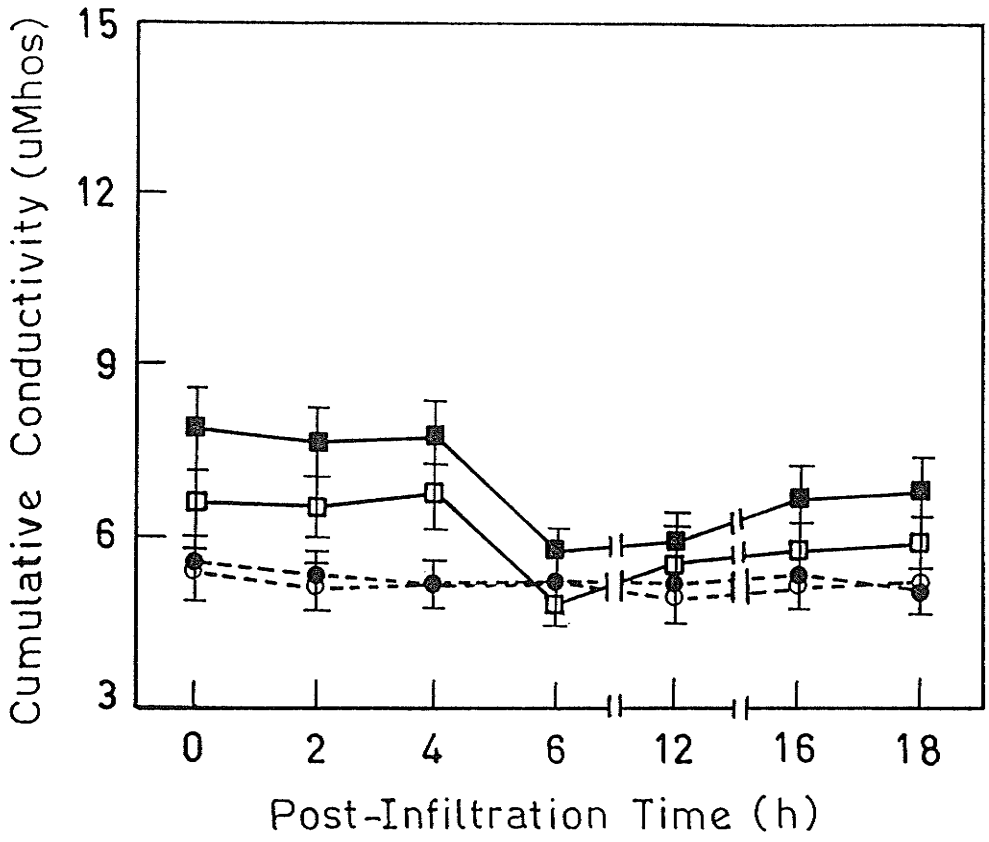


Figure 14. Electrolyte leakage at various post-infiltration times from control and toxin-infiltrated (0.052 µg/ml) leaf tissues of Erik and Glenlea. Leakage was determined by measuring changes in electrical conductivity of the leaching solution in which the tissue was suspended and shaken. Bars represent standard error, and each point is a mean of four samples

Glenlea control

Glenlea toxin-infiltrated

Erik control

Erik toxin-infiltrated

A somewhat different pattern was observed when the Ptr necrosis toxin was used at lower concentrations of 0.52 and 0.052 μg/ml (Fig 13 and 14). The cumulative losses in electrolytes were not statistically different (5% level) from the corresponding controls at all the post-infiltration times studied.

Losses in electrolytes as measured by the cumulative conductivity values from the toxin-insensitive Erik leaves were not dependent upon the time-elapsed after the infiltration of the Ptr necrosis toxin in the leaf tissue. However, losses in electrolytes from the toxin-sensitive Glenlea leaves were dependent upon the exposure time.

5.6 Effect of Ptr necrosis toxin on protein synthesis

The toxin-sensitive Glenlea leaves were infiltrated with sodium acetate buffer and 5.2 μg/ml toxin, respectively. For this study, Glenlea leaves were examined at 6, 12, 18 and 24 h post-infiltration times. To determine the relative level of protein synthesis in infiltrated tissue, leaf segments from the infiltrated zone were vacuum infiltrated with ^{35}S-methionine. The leaf segments were incubated for 1 h to allow the label to be incorporated. The level of incorporation of ^{35}S-methionine into total leaf protein as well as the individual protein synthesis pattern were examined. Total protein was extracted and the incorporation of label into protein was estimated as TCA precipitable counts as outlined in the Methods section. Proteins labelled with ^{35}S-methionine were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorographic techniques.

The incorporation of the methionine label into the TCA-precipitable protein from the control and toxin-treated Glenlea leaves at four post-infiltration times is

summarized in Table 6. At 12, 18 and 24 h post-infiltration times, the amount of label incorporated in the buffer-infiltrated controls was 78%, 76% and 40% of that incorporated at 6 h. The amount of label incorporated into the TCA-precipitable protein fraction in Glenlea toxin-infiltrated leaves was not significantly different from that of the corresponding controls at 5% level (Table 6).

SDS-PAGE patterns of the high molecular weight proteins in the toxin-infiltrated leaves were essentially identical to their respective controls at 6 and 12 h (Fig 15). The 18 h and 24 h toxin-treated samples, however, showed some differences in the protein patterns. The intensity of the subunits of approximately 22 K molecular mass was lower in the toxin-treated samples as compared to their respective controls. Similarly, a decreased intensity of the polypeptide of approximately 14 K molecular mass was observed in the toxin-infiltrated sample of 18 h. Most noticeable was the absence of the polypeptide corresponding to apparent molecular mass of approximately 14 K in toxin-infiltrated 24 h sample. It should, however, also be noted that this particular 14 K subunit was not detected in both the control and toxin-infiltrated samples examined 12 h post-infiltration. Little variation was observed among the other bands seen on the SDS-PAGE gel.

5.7 Effect of Ptr necrosis toxin on leaf chlorophyll content

The possible effects of Ptr necrosis toxin on chlorophyll content were evaluated in both toxin-insensitive, Erik, and toxin-sensitive, Glenlea cultivars. A high toxin concentration (5.2 μg/ml) was used to ensure that all of the infiltrated region of the leaf would experience saturating effects of the toxin. Leaves of both Erik and Glenlea

Table 6. TCA precipitable label extracted from leaf tissue in relation to post-infiltration time of toxin and buffer infiltrated leaves.

	cpm / mg fresh weight of the sample	
Post-infiltration time (h)	Control	Toxin (5.2 μg/ml)
6	51,186	46,658*
12	39,819	28,302*
18	38,940	30,014*
24	20,466	16,193*

Each value represents mean of three samples corrected for fresh weight of the tissue.
* Not statistically different from the corresponding controls (determined from contrast statements)

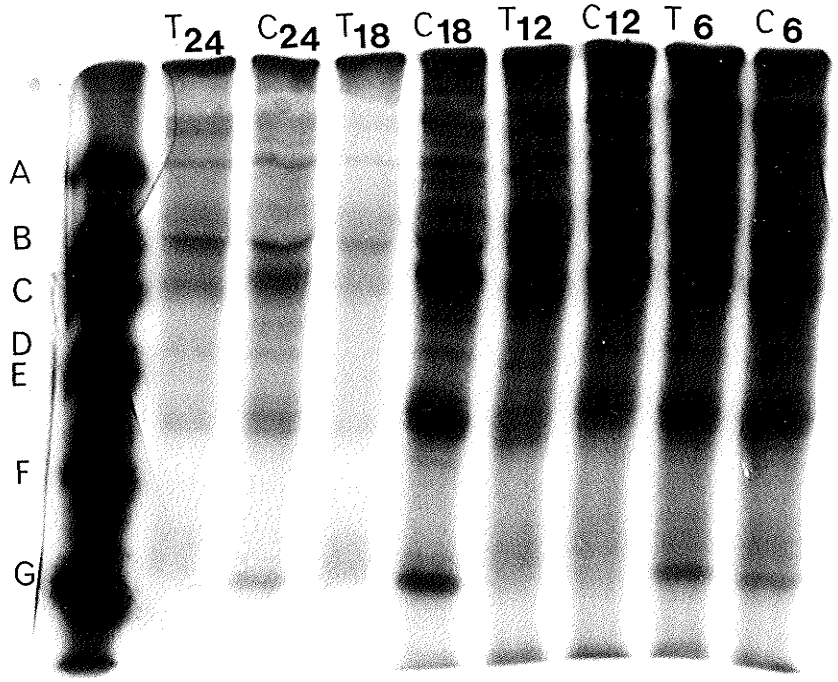


Figure 15. Protein patterns from Glenlea leaves infiltrated with 10 mM sodium acetate buffer and Ptr necrosis toxin (5.2 µg/ml). The infiltrated leaves were labelled with ^{35}S-methionine. Proteins were separated on a 12.5% SDS gel. The letters 'A-G' represent ^{14}C-molecular weight markers (66, 45, 36, 29, 23.6, 20.1, 14.2 K, respectively).

C_6, C_{12}, C_{18}, C_{24} represent the control treatments.
T_6, T_{12}, T_{18}, T_{24} represent the toxin-infiltrated Glenlea leaves.
Counts/min for control treatments and toxin-treated tissues at 6, 12, 18 and 24 h are 62,500; 50,000; 45,454; 23,437; 76,923; 43,478; 20,833 and 27,778, respectively.

were infiltrated with sodium acetate buffer or Ptr necrosis toxin prepared in buffer. Equal number of leaf tissues (four 1 cm leaf pieces) were harvested at 6, 12, 18, 24 and 48 h after infiltration for Glenlea, while for Erik, leaf tissues were harvested only at 6, 12, 18 and 24 h after infiltration. Leaves were extracted with DMF and the amount of chlorophyll was measured spectrophotometrically. The total chlorophyll content of the leaf tissue was expressed as $\mu g/cm^2$ leaf area. Similarly, the chlorophyll a/b ratio was estimated for both the cultivars.

The total chlorophyll content of the buffer-infiltrated controls as well as toxin-infiltrated Erik leaves were not statistically different (5% level) from each other at all post-infiltration times (Fig 16). Similarly, the chlorophyll a/b ratio (Table 7) of toxin-infiltrated Erik was not statistically different from the corresponding controls at all the times studied. By comparison, Glenlea leaves infiltrated with buffer alone showed more variable chlorophyll content, ranging from 32-45 $\mu g/cm^2$, over the post-infiltration times examined. In Glenlea leaves, an increased chlorophyll content relative to the corresponding controls was observed at 6 h after toxin infiltration (Fig 17). However, differences in the chlorophyll content of the toxin-infiltrated samples at 12 and 18 h post-infiltration times were not significant when compared to the corresponding controls.

At 24 h and 48 h, toxin-infiltrated leaves contained significantly less chlorophyll (33% and 36% respectively) than that of the controls. The chlorophyll a/b ratio (Table 7) of the toxin-treated samples was not statistically different from the corresponding controls at any of the post-infiltration times studied.

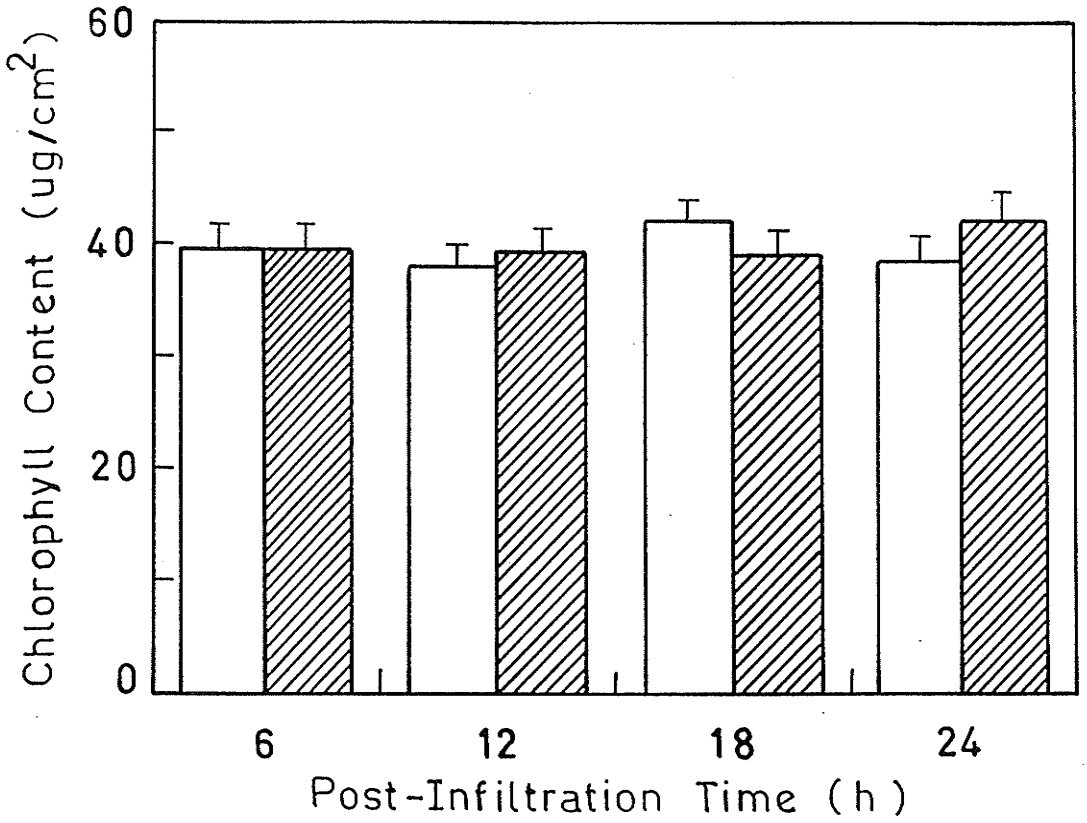


Figure 16. Effect of Ptr necrosis toxin (5.2 μg/ml) on chlorophyll content in toxin-insensitive cultivar, Erik. Bars represent standard error, each value is a mean of three replicates

Control Toxin-infiltrated

Table 7. Chlorophyll a/b ratio of extracts from control and toxin-treated Erik and Glenlea

	Erik		Glenlea	
Post-infiltration time (h)	Control	Toxin[a]	Control	Toxin[a]
6	2.375	2.382*	2.404	2.063*
12	2.351	2.338*	2.377	2.302*
18	2.340	2.361*	2.375	2.256*
24	2.358	2.324*	2.343	2.238*
48	nt	nt	2.484	2.013*

[a]Toxin (5.2 µg/ml)
nt - not tested
* not statistically different from the relative controls at 5% level

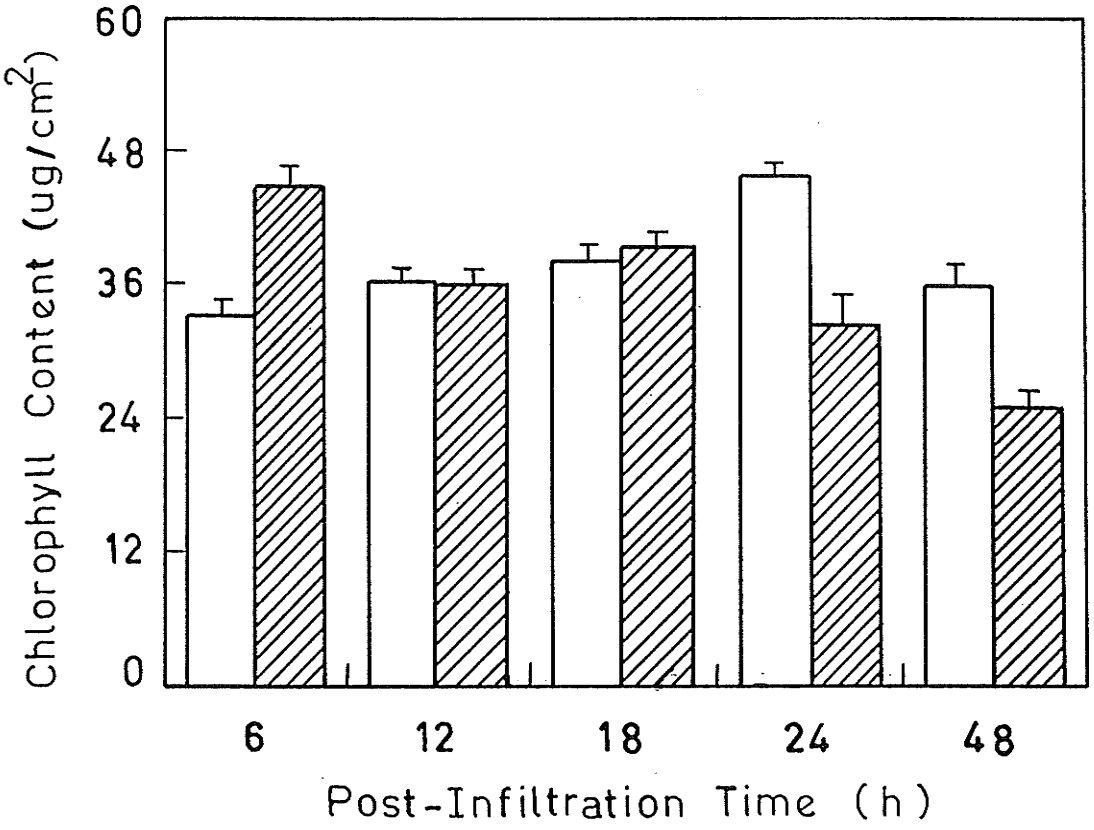


Figure 17. Effect of Ptr necrosis toxin (5.2 µg/ml) on chlorophyll content in toxin-sensitive cultivar, Glenlea. Bars represent standard error, each value is a mean of three replicates

Control Toxin-infiltrated

5.8 Effect of Ptr necrosis toxin on phenolic content

Erik and Glenlea leaves, infiltrated with either sodium acetate buffer as control, or Ptr necrosis toxin, were harvested at 6, 12, 18, 24 and 48 h (Glenlea only) post-infiltration times. To determine the effect of Ptr necrosis toxin on the accumulation of phenolic compounds, the N,N-dimethylformamide extracts of the infiltrated tissues were assayed for total phenolics using the Prussian blue assay. The phenolic content was evaluated on the basis of a catechol standard curve and expressed as μg of catechol equivalents/cm^2 of leaf tissue.

In the toxin-insensitive cultivar Erik, the phenolic contents of the control samples were not statistically different from each other at all post-infiltration times. Similarly, the phenolic content of the toxin-infiltrated Erik leaves did not differ significantly from the respective control treatments at the various post-infiltration times investigated (Fig 18).

The total phenolic content of Glenlea control samples were more variable but not significantly different from each other at all infiltration times. As with Erik, in the toxin-infiltrated Glenlea leaves, phenolic content did not differ significantly from the respective control treatments at 6, 12, 18, 24 and 48 h post-infiltration times (Fig 19).

A major difference between the two cultivars was that the phenolic content of Erik leaves appeared to be quite high compared to that of Glenlea leaves. The phenolic content of the control samples ranged from 200-237 μg catechol equivalents/cm^2 for Erik as compared to only about 40-47 μg catechol equivalents/cm^2

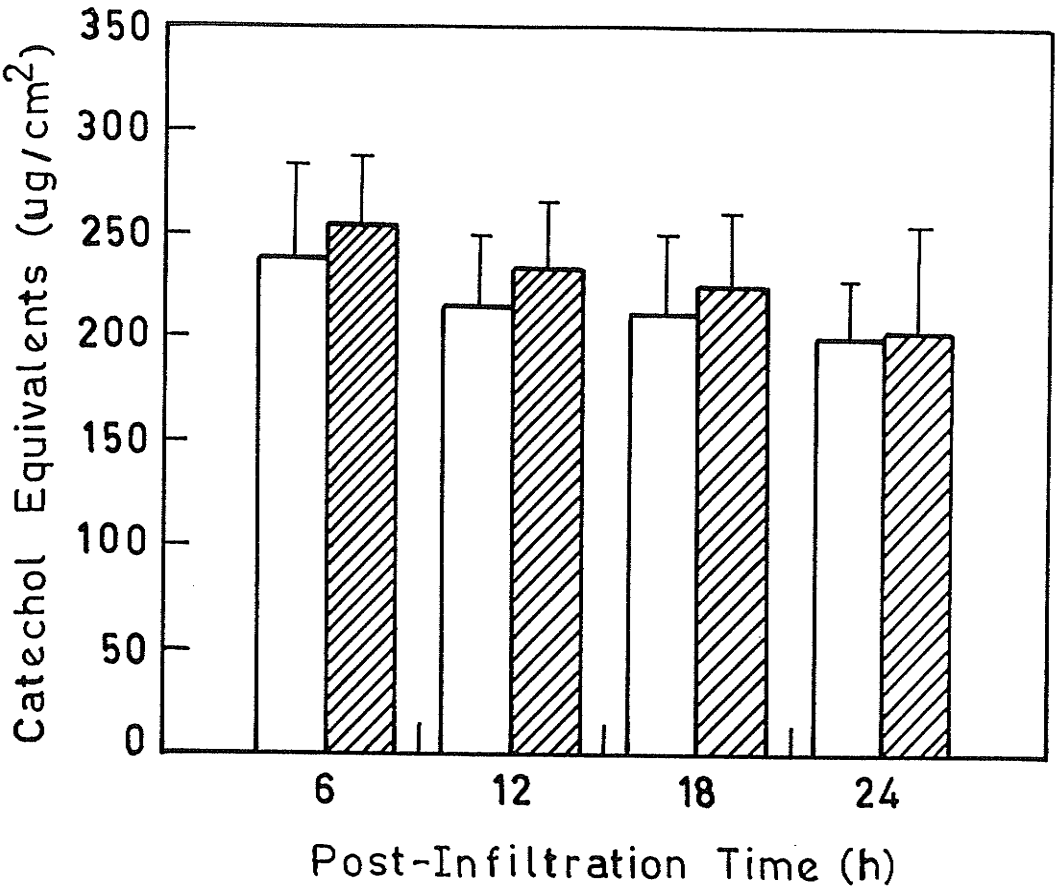


Figure 18. Effect of Ptr necrosis toxin (5.2 µg/ml) on phenolic content in toxin-insensitive cultivar, Erik. Bars represent standard error, each value is a mean of three replicates

Control Toxin-infiltrated

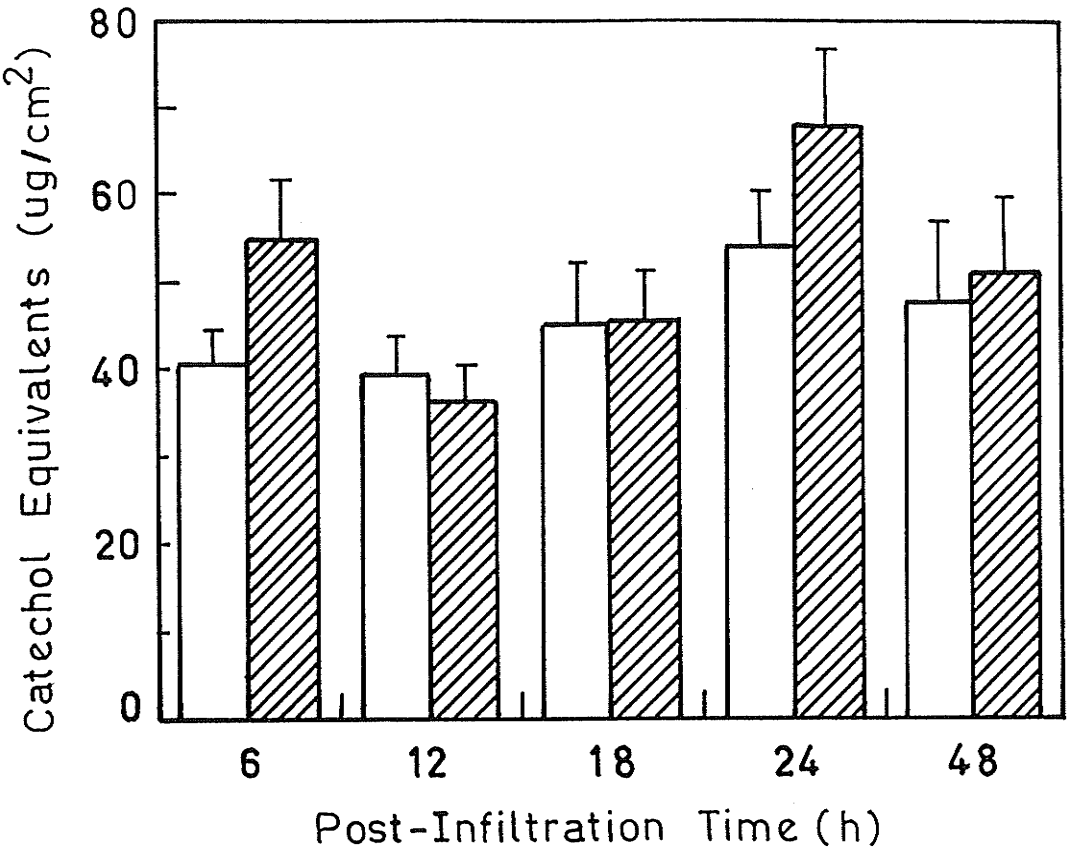


Figure 19. Effect of Ptr necrosis toxin (5.2 µg/ml) on phenolic content in toxin-sensitive cultivar, Glenlea. Bars represent standard error, each value is a mean of three replicates

Control Toxin-infiltrated

of leaf tissue for Glenlea. Thus, Erik leaves appeared to contain 4-5 times higher phenolics than the toxin-sensitive Glenlea leaves.

6. DISCUSSION

6.0 Ptr necrosis toxin

SDS-PAGE analysis of the toxin indicated, a single protein constituent in the sample without any detectable contaminants. The protein constituent corresponded to a molecular mass of 13900 ± 500. Ballance et al. (1989) and Tomas et al. (1990) independently reported the purification of the host-selective Ptr necrosis toxin. They characterized the toxin as a protein with molecular mass of 13,900 ± 500 and 14,700 respectively. It was concluded that the toxin purified in the present study was free of detectable contaminants.

The purified toxin when infiltrated into the toxin-insensitive cultivar at a concentration of 52 μg/ml did not cause any necrosis. Infiltration at the same concentration into the toxin-sensitive cultivar produced visible necrosis as early as 18 h and killing the entire infiltrated zone by 72 h. The toxin isolated in this study was highly potent and active at low concentrations similar to that isolated by Ballance et al. (1989).

6.1 Cell suspension culture

The suspension cells initiated from established calli were monitored for viability, in the presence and absence of toxin, for a period of 48 h. Toxin-treated suspension cells from Erik and Glenlea were not affected by the toxin when examined up to 48 h. Cell viability beyond 48 h was not assessed. However, a longer exposure time might have produced an effect on the suspension cells.

The toxin concentration selected for the cell suspension study was based on the observations from the leaf infiltration work. In the latter work, a toxin concentration of 0.52 μg/ml produced visible necrotic symptoms in leaf tissue after 24 h. However, at neither 3.5 nor 6.7 times this concentration was any visible change observed for the cells in suspension culture. Since cells in culture are undifferentiated and non green, perhaps, such cells are less responsive to the toxin.

6.2 Callus culture

To examine the effect of Ptr necrosis toxin on callus induction, replicated plates of calli from toxin-sensitive and insensitive cultivars were allowed to grow and differentiate on toxin-containing and toxin-free media. Glenlea and Erik calli, on toxin-free medium, responded well in culture and formed normal shoots and roots. Similarly, growth of Erik calli was not affected by the presence the toxin, with the shoot and root development not significantly differing from that of the controls. Growth of Glenlea calli prior to differentiation on toxin-containing media was not inhibited by the toxin (21 μg/ml), and they grew similarly to their respective controls. However, beyond the initial callus growth phase, there was a significant inhibition of the shoot development and a stimulation in root formation on toxin media.

In the present investigation, toxin inhibition of callus growth was not observed. As pointed out before, necrotic symptoms are detectable as early as 24 h in the leaf infiltration assay when infiltrated at 5.2 μg/ml toxin concentration. Based on the cell suspension culture results and the fact that callus is also composed of undifferentiated cells, it can be suggested that undifferentiated cells are insensitive to the toxin and that

the toxin sensitivity may be a tissue-specific response in certain differentiated tissues.

Inhibition in the growth of the callus is a commonly observed phenomenon in the case of many low molecular weight toxins (Wolf and Earle, 1990; Gengenbach and Green, 1975; Pauly et al., 1987). The only pronounced effect of the Ptr necrosis toxin observed was the inhibition of shoot and stimulation of root formation in Glenlea calli. Inhibition of both shoot and root formation in the presence of toxin has been reported in corn calli in response to HC-toxin (Wolf and Earle, 1990). In the present study, however, a stimulation in root formation was observed with some of the sensitive calli on toxin-containing media after considerable exposure to the toxin.

It is a well known fact that increased ethylene production is characteristic of numerous fungal, viral and bacterial diseases (Hislop and Stahmann, 1971). The auxins and cytokinins used in tissue culture stimulate ethylene production (Garcia and Einset, 1983; Gavinlertvatana et al., 1980). Ethylene induced abnormal shoot growth in shoot cultures of potato (Creissen and Karp, 1985) and promoted shoot or root regeneration in tissue cultures of rice (Cornejo-Martin et al., 1979). Thus inhibition of shoots and stimulation of root formation, with massive proliferation in some of the susceptible calli, in the present study may largely be due to toxin induced ethylene production in the medium.

Although the toxin produced a differential response on the organ differentiation in the toxin-sensitive calli, perhaps due to ethylene production by the tissue, ethylene production was not analyzed in the present study. Further studies are required to demonstrate whether increased ethylene is present in toxin-treated Glenlea calli.

6.3 Electrolyte leakage

To investigate the effect of Ptr necrosis toxin on the loss of electrolytes in Erik and Glenlea cultivars, the plants were infiltrated with dilute sodium acetate buffer and various concentrations of toxin in the same buffer, respectively. Random 1 cm sample pieces (65 pieces) of infiltrated zones were harvested at 0, 2, 4, 6, 12, 16 and 18 h post-infiltration times, and electrolyte losses were measured.

A steady leakage of electrolytes was observed in both the control and toxin-infiltrated (5.2 μg/ml) Erik leaves over a 60 min leaching period when measured at 0 h and 18 h post-infiltration times. Ptr necrosis toxin consistently failed to show any adverse effect on the loss of electrolytes in toxin-insensitive leaf tissue.

At 0 h post-infiltration time and at 5.2 μg/ml toxin, the leakage rate of the toxin-infiltrated Glenlea tissue, was similar to the control. However, at 18 h, a significant increase in the leakage was observed during the initial 30 min of the total 60 min. Compared to this, similar data at a lower toxin concentration (0.52 μg/ml) indicated smaller electrolyte losses. The loss of electrolytes was, therefore, dependent upon the concentration as well as the time of exposure to the toxin in toxin-sensitive tissue. At 5.2 μg/ml toxin concentration, the greatest losses in electrolytes were observed during 16-18 h period in toxin-infiltrated Glenlea.

It appears from the results presented in Figures 12-14 that the toxin requires at least 16-18 h resident period within the leaf tissue to reach its target site. In contrast, low molecular weight host-selective toxins produced by *Alternata alternata* (Namiki et al., 1986), *Helminthosporium victoriae* (Wheeler and Black, 1963; Luke et al.,1969;

Schroeter et al., 1985), *Periconia circinata* (Scheffer, 1976), *H. carbonum* (Comstock and Scheffer, 1973), *Alternaria Kikuchiana* (Otani et al., 1973), *A. mali* (Kohmoto et al., 1976) and *H. maydis* (Gracen et al, 1972) induced a rapid increase in loss of electrolytes as well as depolarized the membrane of toxin-sensitive genotypes indicating plasma membrane as the target site.

The increase in the loss of electrolytes from toxin-sensitive tissue was also dependent on the toxin concentration. Wheeler and Black (1963) observed that the rate of electrolyte leakage increased when the sensitive oat tissue was exposed to increasing concentrations of victorin, the causal agent of Victoria blight of oats. Larkin and Scowcroft (1981) also observed similar toxin concentration dependence of the electrolyte leakage for the host-selective toxin produced by *Helminthosporium sacchari* that causes the eyespot disease of sugarcane. The host-selective toxin produced by *H. maydis* race T causes leakage of electrolytes from root and leaf tissue in susceptible corn genotypes, the rate of which was dependent upon both the temperature and time of exposure to toxin (Halloin et al., 1972).

The evidence from the literature thus suggests that damage to cell membranes upon exposure to host-selective pathotoxins occurs within a relatively short period of time and that plasma membranes are the primary site of action in many instances. In contrast, the time course study of the loss of electrolytes in the present investigation indicated that a period of at least 16-18 h exposure was essential for the toxin to exert its toxicity in the sensitive host tissue. Such differences may be related to the molecular mass of the host-selective toxins from differing sources. Unlike victorin

and helminthosporoside, which are small molecular weight toxins (molecular masses of less than 1000), the Ptr necrosis toxin is a relatively large toxin (13,900 ± 500). This ultimately would result in longer time for the observed effects on cell membrane permeability for the Ptr necrosis toxin, as compared to the exposure times required for other host-selective toxins such as victorin and helminthosporoside (Wheeler and Black,1963; Larkin and Scowcroft, 1981).

It should also be noted that the Ptr necrosis toxin is unable to penetrate the leaf tissue by simple diffusion process. Hence, the toxin had to be infiltrated into the leaf tissue to observe its effects on electrolyte leakage.

Several researchers have suggested the possibility of receptor sites for host-selective toxins to be located on the plasmalemma of susceptible host cells. This hypothesis was primarily based on the fact that changes in the permeability of plasmalemma occur in cells soon after toxin treatment (Samaddar and Scheffer, 1968, 1971; Novacky and Hanchey, 1974), and that wall-less protoplasts lyse after exposure to victorin toxin (Samaddar and Scheffer, 1968). The observations of Strobel (1974b) also suggest a preferential binding of the HS toxin *in vivo* to tissues of susceptible but not resistant sugarcane cultivars. He further observed that a protein isolated from resistant clones that was immunologically similar to the one isolated from the susceptible clones was unable to bind to the toxin. Subsequent studies, however, have contradicted these observations (Yoder, 1980; Daly, 1981).

Using a similar rationale, it is quite likely that in susceptible wheat cultivars such as Glenlea, the plasma membranes contain sites that are sensitive and bind to the

toxin *in-vivo*, which ultimately leads to drastic alterations in cell membrane permeability causing cell death and necrotic lesions. If this hypothesis were to be true, then the resistant or toxin-insensitive wheat cultivars may contain proteins or other factors that prevent the binding of the Ptr necrosis toxin to the cell membrane. Corollary, the toxin may directly affect the plasma membrane H^+- ATPase (Marre, 1980) affecting the activity of a number of metabolic and physiological processes.

Cell membrane appears to be the site of action of the Ptr necrosis toxin in the toxin-sensitive Glenlea cultivar. The damage to cell membranes of the toxin-sensitive wheat cultivars appears to be primarily dependent upon the time elapsed after the infiltration of the toxin.

6.4 Incorporation of ^{35}S-methionine and protein synthesis

To determine the effect of Ptr necrosis toxin on the inhibition or alteration of protein synthesis, buffer-infiltrated and toxin-infiltrated Glenlea leaves were labelled with ^{35}S-methionine. Incorporation of the label into protein was estimated by TCA-precipitable counts and SDS-PAGE patterns of the incorporated proteins were visualized by fluorographic techniques as outlined in the Methods section.

As the effect of Ptr necrosis toxin on protein synthesis was only a preliminary study, the toxin-insensitive cultivar Erik was not used as a reference control sample in this series of experiments.

The incorporation of label into proteins in the control treatments was variable at all post-infiltration times. This variability could be due to the inherent heterogenous nature of the leaf tissue as well as the experimental technique (vacuum infiltration of

the label) employed. These reasons may also have contributed to the apparent non-labelling of the 14 K subunit in both the control and toxin-infiltrated samples of the 12 h post-infiltration treatment. With the exception of 12 h, SDS-PAGE patterns of the incorporated label into proteins of the control treatments were similar at all post-infiltration times.

Contrary to expectation, the incorporation of label into toxin-infiltrated Glenlea proteins was not different from the corresponding controls at all the post-infiltration times studied. The protein patterns of the toxin-infiltrated leaves were similar to their respective controls at 6 and 12 h. However, at 18 and 24 h, a decrease in the intensity of the protein bands corresponding to 14 K and 22 K was observed.

Samaddar and Scheffer (1968) reported a low ^{32}P uptake by victorin-treated sensitive oat leaf tissue as compared to the corresponding control, and suggested that the low ^{32}P uptake during protein synthesis in the toxin-treated tissue was perhaps a secondary effect of the victorin toxin as leakage of electrolytes was well pronounced within 20 min.

As compared to host-selective pathotoxins, several mycotoxins, particularly those belonging to the trichothecene group (e.g., deoxynivalenol or vomitoxin, and T-2 toxin) as well as aflatoxins produced by *Aspergillus flavus*, are known to act directly on the DNA expression (Sporn et al., 1966; Casale and Hart, 1988). In the only instance where a plant pathotoxin was implicated to affect the protein synthesis, Penner et al. (1969) originally proposed that syringomycin interfered with DNA-dependent RNA synthesis. Subsequent studies by Backman and DeVay (1971),

however, have shown this to be a secondary effect resulting due to alterations in cell membrane permeability.

Stress responses sometimes also induce unique protein patterns similar to those in infected plants (Uritani, 1970). Only two protein bands appeared to be affected in the toxin-treated Glenlea leaves after 18 and 24 h exposure. Daly (1981) suggests that with the experimental techniques currently available to detect changes in protein patterns, only the major dramatic effects can be studied.

In the present study, the Ptr necrosis toxin failed to show any dramatic effect on the incorporation of radiolabelled methionine as well as on the protein patterns observed by fluorographic SDS-PAGE gels. As compared to the adverse effects on the cell membrane permeability, Ptr necrosis toxin exerted only minor effects on radiolabel incorporation and protein synthesis, thereby suggesting a secondary effect.

6.5 Chlorophyll content

To determine the effect of Ptr necrosis toxin on chlorophyll content, chlorophyll was extracted from buffer and toxin-infiltrated Erik and Glenlea leaves at 6, 12, 18, 24 and 48 h post-infiltration times. The fresh weight of the toxin-infiltrated tissue declined considerably after 18 h post-infiltration time, and hence, the total chlorophyll content was measured on a leaf area basis rather than the fresh weight. Total chlorophyll content was estimated and expressed as $\mu g/cm^2$. Similarly, chlorophyll a/b ratio was estimated in the tissue extracts from both cultivars.

The total chlorophyll content of toxin-infiltrated Erik leaves was not different from the corresponding controls at all post-infiltration times. Glenlea leaves infiltrated

with buffer showed much greater variability in chlorophyll content at all infiltration times. In toxin-infiltrated Glenlea leaves, the total chlorophyll content decreased after 24 h exposure to the toxin. However, the fact that necrotic lesions normally begin to appear by 24 h at the toxin concentration used in the present study indicates that the decrease in chlorophyll content is a late event and is possibly a consequence and not a cause of cell death. If chloroplasts were to be the primary target site of the Ptr necrosis toxin, the changes in chlorophyll content should have been evident at an earlier time, and well before the appearance of necrotic lesions on the leaf tissue. This certainly was not the case in the present study.

Certain non-host-selective toxins, however, are known to inhibit chlorophyll synthesis which is considered to be one of the modes of action of these toxins. The best known example is perhaps tentoxin, produced by *Alternaria tenuis.* This toxin causes chlorotic symptoms in cucumber seedlings through disruption of normal plastid development rather than through direct interference with chlorophyll synthesis (Halloin et al., 1979) at 96 h after incubation in the toxin. Tabtoxin (wildfire toxin), produced by *Pseudomonas syringae pv. tabaci*, and T-toxin are also known to interfere with the formation of photosynthetic pigments, thereby causing extensive chlorosis of the susceptible host leaf tissue (Crosthwaite and Sheen, 1979; Bhullar et al., 1975; Daly, 1981). These chlorotic symptoms were generally related to one of the following four mechanisms: (1) inhibition of chlorophyll synthesis, (2) degradation of chlorophyll, (3) disruption of plastids, and (4) possible inhibition of RUBISCO enzyme systems.

The effects of Ptr necrosis toxin on the chlorophyll content of toxin-treated leaves thus appeared to be a secondary effect to that observed on the electrolyte leakage. The primary reason for this conclusion is the fact that up to 18 h after exposing the leaf tissue to the toxin, very little change took place in the chlorophyll content of the toxin-sensitive leaf tissue. In contrast, by this time, a significant effect had already taken place on cell membrane permeability as was evident by the leakage of electrolytes from cell cytoplasm.

6.6 Phenolic content

To examine the effect of Ptr necrosis toxin on the accumulation of phenolic compounds, triplicate analyses of DMF extracts were assayed using Prussian blue assay. The toxin did not have any affect on the phenolics levels in both cultivars relative to the controls at the various infiltration times. However, the toxin-insensitive cultivar Erik had comparatively higher phenolic levels than Glenlea.

Phenolic compounds of phytoalexin group, which accumulate at appropriate times and places, are well known to be involved in disease resistance mechanisms in several plants (Bailey, 1982; Deverall, 1982; Mansfield, 1982). Several examples of varieties of plant species being more or less resistant to pathogens depending upon their content of natural tannin or other phenols are reported in the literature (Harris and Burns, 1973; Kuc, 1966). These examples suggest that the general response to attack by pathogens commonly involved production of high levels of phenols and an increase in the activity of phenolase enzyme complex.

One interesting feature of the present study on the phenolic content of Glenlea and Erik leaves was the fact that the toxin-insensitive Erik contained nearly 4-5 times higher phenolics. Although different phenolic compounds differ in their ability to reduce the ferricyanide complex in the Prussian blue or similar redox assays used for the determination of the phenolic compounds (Price and Butler, 1977), such a large difference in the phenolic compounds of Erik and Glenlea leaves certainly cannot be explained by this fact alone. While the phenolic content did not change at any of the exposure times used in this experiment, the possibility that the inherently higher phenolic content of Erik may play an important role in resistance response cannot be disregarded. Phenolic compounds are also well known to form complexes with different proteins and enzymes, thus interfering with their normal physiological functions. Additional studies on the nature and types of phenolic compounds present in the leaves of toxin-sensitive and toxin-insensitive wheat leaves are required. Similarly, the role of phenolic compounds in the induction of resistance response to the pathogen as well as insensitivity to the Ptr necrosis toxin needs to be elucidated.

7. CONCLUSIONS

1. Ptr necrosis toxin did not affect cell viability in suspension cell cultures.
2. The toxin did not produce any effect on the growth of the calli, either in the toxin-insensitive or toxin-sensitive plant tissue.
3. The toxin inhibited shoot development and stimulated root formation in toxin-sensitive calli.
4. The toxin induced differential leakage of electrolytes in toxin-sensitive tissue. The earliest detectable response was observed 16 h post-infiltration. The plasma membrane, therefore, may be the potential site for electrolyte leakage in toxin-sensitive tissue.
5. Protein synthesis and chlorophyll contents were not affected by the toxin, but only at the time when visible necrosis was observed.
6. Ptr necrosis toxin did not produce any detectable effects on phenolic content.
7. The higher levels of phenolics observed in toxin-insensitive cultivar may be associated with its toxin-insensitivity.

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