THE ROLE OF *PYTHIUM* AS GLYPHOSATE SYNERGISTIC FUNGI

by

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Abstract

Glyphosate synergistic interaction (GSI) refers to the enhanced efficacy of sublethal doses of glyphosate to plants in the presence of certain soil fungi. Information on the role of *Pythium* in GSI is lacking, therefore, the following topics were investigated: *Pythium* species capable of GSI and their distribution in soils; host-specificity of glyphosate synergistic *Pythium* (GSP); virulence of GSP to healthy seedlings in the absence of glyphosate; relationship of characteristics of *Pythium* such as *in-vitro* growth and virulence to an isolate's efficacy as a synergist; and the effects of glyphosate-treated plants on the populations of natural and introduced *Pythium* in soil.

Thirty-nine *Pythium* isolates representing 14 species were tested for GSI on 2-week-old bean seedlings. To identify GSP, LD_{50} values for glyphosate on seedlings grown in sterilized soils (control) and in soils amended with each isolate were separately estimated. The magnitude of differences in LD_{50} between *Pythium*-amended and control soils varied between species and among isolates within species. The GSP were widely distributed in four agricultural soils and one virgin soil. No host-specificity as glyphosate synergists was found among the isolates obtained from roots of glyphosate-treated bean and wheat plants, or among isolates from various untreated hosts. Most isolates tested were pathogenic to varying degrees on germinating seeds of wheat, corn, bean, pepper and sunflower. *In-vitro* growth and virulence by the various isolates were poorly correlated with their efficacy as synergists.

Natural *Pythium* populations in organic soil, and populations of individual GSP and non-GSP introduced into sterilized soil, were

increased by foliar application of herbicides to bean seedlings growing in the soil, or by amendment of the soil with heat-killed bean roots. Strong positive correlations were observed between population estimates for *Pythium* obtained by dilution plating of these soils and damping-off in sunflower seedlings which indicate that herbicide treatment of plants may cause temporary increases of *Pythium* populations and damping-off in soils.

The research shows that diverse *Pythium* isolates can enhance the efficacy of a sublethal glyphosate dose. The role of *Pythium* species in GSI was attributed to their inherent character as opportunistic and pioneer colonists of virgin organic substrates in soil.

Dedication

To Delia and R.G. Kirby Descalzo,

for making my life complete...

R.D.

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Chapter I

GENERAL INTRODUCTION

The subject of this thesis is the study of interactions that occur in glyphosate-treated plants between the herbicide glyphosate and members of the soilborne fungus *Pythium*. In studying complex relationships such as this, it is necessary to have adequate background information about the major factors involved in the interaction. This information is needed to formulate hypotheses and for selection of appropriate methodology and techniques required to carry out the experiments. Relevant literature reviews on glyphosate and *Pythium* are presented in the following two sections.

Section 1. Glyphosate

Glyphosate Development History

In the late 1960's, a search for herbicides that were effective at low rates and environmentally safe became a major goal of various chemical companies. To develop a herbicide with these desirable characteristics, researchers looked for compounds that only affected the biochemical pathways unique to plants. One such compound, glyphosate (N-[phosphonomethyl] glycine), was discovered in 1971 by researchers at Monsanto Agricultural Products Co. (Baird *et al.*, 1971).

Many unique physical, chemical and biochemical properties of glyphosate made it one of the most widely used broad spectrum, non-selective, post emergence herbicides in the world. Glyphosate is a water soluble herbicide which is readily absorbed through foliar tissues and is

translocated to the meristematic regions of plants (Gougler and Geiger, 1981). The translocation and accumulation of glyphosate in plant meristems enable it to control perennial weeds that are extensively rooted and vegetatively propagated, such as couch grass (*Agropyron repens*), thistle (*Cirsium arvense*), nutsedge (*Cyperus* spp.), etc. (Sprankle *et al.*, 1975). Negligible mammalian toxicity is the most significant characteristic of glyphosate. The acute toxicity of glyphosate in mammals is less than that of table salt, and long term tests in rats demonstrated no teratogenic, mutagenic or carcinogenic effects (Grossbard and Atkinson, 1985).

Uses of Glyphosate

Glyphosate is sucessfully marketed in 119 countries under various tradenames, such as Roundup®, Vision®, Rodeo® and Kleenup®. It is labelled for use on more than 50 agricultural crops and also for application in industrial and forestry sites (Grossbard and Atkinson, 1985). Its varied use is limited only by its lack of selectivity. In worldwide farming situations, it is sprayed prior to cultivation of fallow land, and in minimum tillage or non-tillage systems, it is applied prior to direct planting of crops (Terry, 1985). Glyphosate has been used for aquatic weed control in many countries (Barrett, 1985), and is also applied as a desiccant to hasten maturation of crops (Whigham and Stoller, 1979). Forestry uses of glyphosate involves late summer applications to control competing vegetation in conifer reforestation areas (Lund-Hoie, 1985).

In Canada, glyphosate is currently registered for pre-plant and post-harvest control of annual and perennial weeds in continous, summerfallow and minimum tillage cropping systems (Spencer, 1973). It

is also used for control of woody brush and trees on rights-of-way, and for directed application in orchards, vineyards and shelterbelts. Glyphosate is used for control of undesired woody and herbaceous species for site preparation, and conifer release in forestry and woodland sites and in forest nurseries. Several dilute formulations of glyphosate are registered for home garden use (Doliner, 1991).

Glyphosate was approved for pre-harvest weed control and as a harvest management tool (crop desiccant) in Canada in 1991 (Doliner and Stewart, 1992). With these newly approved uses for glyphosate, some predictions are that as a crop desiccant, glyphosate might be applied to 5 to 10 million hectares of prairie croplands (Rahe *et al.*, 1990).

Mode of Action

Extensive literature reviews on the biochemical basis of glyphosate phytotoxicity have been done by Cole (1985) and Coggins (1989). Elucidation of the target site of glyphosate action in plants began when Jaworski (1972) observed that in *Lemna gibba*, a tiny aquatic plant called duckweed, aromatic amino acid levels were reduced after glyphosate treatment. Jaworski also reported that growth inhibition by glyphosate in duckweed was suppressed by the addition of aromatic amino acids, such as L-phenylalanine, L-tyrosine and L-tryptophan to the medium. Jaworski suggested that since these amino acids were produced from the shikimic acid pathway, glyphosate may have affected a metabolic step in the pathway.

The theory of aromatic amino acid depletion by glyphosate was strongly supported by subsequent *in-vitro* experiments. Gresshoff (1979)

demonstrated the reversal of toxicity by aromatic amino acids in *Escherichia coli, Chlamydomonas reinhardii,* and in tissue culture cells of carrot *(Daucus carrota)* and soybean *(Glycine max).* However, attempts by a number of researchers to reverse glyphosate-induced inhibition of growth within intact plants by amino acid applications were only partially successful (Hoagland and Duke, 1982). The contrasting results from experiments involving intact plants have led to investigations of other possible modes of action for glyphosate. Various researchers have observed that treatment of intact plants with glyphosate affects normal physiological processes, such as chlorophyll synthesis (Hollander and Amrhein, 1980; Kitchen *et al.,* 1981; Lee, 1981), protein synthesis (Cole *et al.,* 1983), auxin production (Lee, 1982), and ammonia accumulation (Cole *et al.,* 1980). While these physiological effects were commonly proposed as part of the mechanism of glyphosate phytotoxicity, some researchers interpreted them to be secondary effects since they were not consistently exhibited by a variety of plants.

Definitive proof supporting the proposed mode of action of glyphosate in plants as suggested by Jaworski in 1982 originated from observations of analogous effects of glyphosate in bacterial cells. Steinrucken and Amrhein (1980) clearly identified the shikimic acid pathway as the specific target site of glyphosate in bacteria. Confirmation of this mode of action for glyphosate came when plant cell cultures and bacterial cultures grown in the presence of moderate concentrations of glyphosate were found to adapt and grow at nearly normal rates by over-expressing genes responsible for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme found in the shikimic acid pathway (Amrhein *et al.,* 1983; Nafziger *et al.,* 1984). Other researchers showed

that glyphosate resistance in bacteria could arise by the acquisition of a glyphosate-resistant form of EPSP synthase by mutation (Comai *et al.*, 1983).

Recently, several researchers have cloned and transferred different forms of EPSP synthase genes from various glyphosate-resistant bacteria into tissue-cultured plant cells to produce glyphosate-resistant transgenic plants (Comai *et al.*, 1985; Fillati *et al.*, 1987). This remarkable result provides definitive evidence that the primary mode of action of glyphosate in intact plants is the inhibition of EPSP synthase. Inhibition of this enzyme prevents formation of the aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan which are essential for protein synthesis. It is now clear that inhibition of EPSP synthase also prevents the formation of precursor compounds needed for secondary metabolism in plants, which explains various other physiological responses observed by researchers in glyphosate-treated intact plants.

Direct Effects of Glyphosate to Soil Microorganisms

Since the shikimic acid pathway is present in microrganisms as well as in plants, it is not surprising that glyphosate also affects microorganisms in soil. Direct *in vitro* toxicity of glyphosate to various saprophytic, pathogenic, and ectomycorrhizal fungi in monoculture have been documented by many researchers, and is summarized in reviews done by Grossbard (1985) and Quinn (1993). A direct comparison of results from various studies is difficult because of the range of media, inocula, cultural conditions, and differences in methodology used in the research. However, it is clear from these studies that glyphosate does exert some inhibitory effects on growth of many fungi, although often at

higher concentrations than would normally be used in the field. However, detailed toxicity studies of commercially formulated glyphosate on plant pathogenic fungi, *Septoric nodorum* (Harris and Grossbard, 1979) and *P. ultimum* and *P. sylvaticum* (Liu, 1995) has shown that the non-active ingredients of the formulation were also inhibitory to fungal growth. These results should be considered in drawing conclusions from the majority of the reported glyphosate toxicity studies on various fungi that uses the commercial glyphosate formulation.

The number of organisms that have been studied with regard to glyphosate sensitivity is less for actinomycetes and bacteria than for fungi. Interpretation and comparison of results from these studies is also difficult, since different methods have been used, and many of the isolates were not identified. Of the few named cultures that have been tested, *Rhizobium japonicum* (Jaworski, 1972), *E. coli* , and *Pseudomonas aureofasciens* (Roisch and Lingens, 1980) were found to be inhibited by various levels of glyphosate *in-vitro*. In some studies, researchers observed that inhibition of bacterial growth by glyphosate did not always cause cell death (Schulz *et al.*, 1984), and pronounced differences between species and isolates were noted (Rogers *et al.*, 1983). These observations have led to studies of mechanisms of glyphosate resistance in some bacteria which resulted in identification of genes responsible for the glyphosate-resistant forms of EPSP synthase that were eventually cloned and used in genetic engineering of glyphosate-resistant plants discussed earlier (Comai *et al.*, 1983).

There are very few reports of glyphosate toxicity to soil algae. Glyphosate was found to affect photosynthesis in *Scenedesmus spp.* (Van Rensen, 1974), *Euglena gracilis* (Richardson *et al.*, 1979), and

Chlorella sorokiniana (Christy *et al.*, 1981). It has been suggested that an algal-based bioassay could be a sensitive tool for quantification of residual glyphosate in the soil (Richardson *et al.*, 1979).

Indirect Effects of Glyphosate to Soil Microorganisms:

Some of the glyphosate applied in field situations to kill weeds may reach the soil through root exudation (Coupland and Caseley, 1979) as well as by direct deposition on the soil surface. Glyphosate is adsorbed tightly by soil particles (Glass, 1987), and for this reason microorganisms which are generally inhibited by glyphosate in pure culture on laboratory media may not be affected in soil. The numbers of propagules of fungi and bacteria in soil were commonly observed to increase for certain periods following glyphosate treatment (Rueppel *et al.*, 1977; Chakravarty and Chartarpaul, 1990). This observation implies utilization by soil microflora of either glyphosate itself or its degradation products (Roslycky, 1982). Inhibitory effects of glyphosate were observed in cellulytic fungi in soil, but inhibition occurred only at concentrations well above those normally used in agriculture (Grossbard, 1985).

Interactions with plant pathogens. Interrelations between herbicides and plant pathogens are complex. Literature reviews of herbicide and plant disease interactions have been done by Altman and Campbell (1977); Altman and Rovira (1989); and Lévesque and Rahe (1992). Many herbicides, including glyphosate, may inhibit growth, spore formation, germination and aggressiveness of plant pathogens, properties which might be utilized for disease control. Unfortunately, too few data are available to permit a general conclusion about the potential of

glyphosate for inhibiting spore production by pathogens on treated plant residues (Grossbard, 1985). The few documented examples of glyphosate inhibitory effects on spore formation in pathogenic fungi on treated plants were observed in *Rhyncosporium secalis* on barley (Grossbard, 1985) and *Pyrenophora tritici-repentis* on greenhouse-grown wheat (Sharma *et al.*, 1989).

In contrast, a relatively large number of reports have indicated the potential of glyphosate-killed plants for providing a general nutrient source for microorganisms, including plant pathogens. When glyphosate was applied as a crop desiccant, it provided fungal pathogens with a suitable substrate for colonization, and consequently may have furnished inoculum for re-infection or further primary invasion (Whigham and Stoller, 1979; Cerkaukas *et al.*, 1982). Glyphosate could also influence re-infection by controlling weed hosts and volunteer plants. Eradication of such plants might be beneficial if they act as alternate hosts. Conversely, dead plants could be harmful by making plant residues available as substrates for colonization. Stedman (1982) showed that volunteer plants of barley, killed by paraquat or glyphosate, served as a source of inoculum for *R. secalis*. Another known indirect interaction was the enhanced development of *Fusarium culmorum* on plant hosts weakened by acetic acid that were exuded from dead or dying rhizomes of *Agropyron repens* killed by glyphosate (Lynch and Penn, 1982).

Other examples of indirect interaction of glyphosate have been associated with the use of glyphosate in agricultural areas. In Western Australia, extensive crop losses occurred following the use of glyphosate (Roundup®) immediately before the direct drilling of cereals; these losses were largely attributed to a seedling root rot caused by *Pythium* which

built-up to high levels on pasture residues after glyphosate use (Blowes, 1987). Research from Oregon State has demonstrated that *Rhizoctonia solani* can be a major problem in no-till spring barley when glyphosate is used to kill weeds 2 or 3 days before planting, but not when it is applied 3 weeks before planting or when it is used 1 or 2 days after direct drilling of spring barley (Smiley *et al.*, 1992).

The interactions of herbicides and fungal root pathogens with special reference to glyphosate was reviewed recently by Lévesque and Rahe (1992). Their review summarized the most recent evidence of the impact of predisposition of plants to root diseases by glyphosate. This interesting indirect effect of glyphosate involves the interaction of three main factors, namely: glyphosate, plant host and soilborne microorganisms. The mechanism of predisposition of glyphosate-treated plants to foliar, stem and root pathogens seems to be well documented. Glyphosate was found to block the production of the phytoalexin glyceollin in soy beans inoculated with the stem and root-infecting fungus, *Phytophthora megasperma* f. sp. *glycinea* (Keen *et al.*, 1982) or with a leaf-infecting bacterium, *Pseudomonas syringae, p.v. glycinea* (Holliday and Keen, 1982). Their results demonstrated that inhibition of glyceollin production rendered soy bean plants susceptible even to incompatible strains of these pathogens. The production of glyceollin utilizes phenylalanine and tyrosine as precursors. Pre-feeding glyphosate-treated soy beans with these two aromatic amino acids restored glyceollin production and total expression of resistance to the fungus and partial resistance to the bacterium.

A similar mechanism of glyphosate-induced disease predisposition of bean hypocotyls was reported by Johal and Rahe (1990). They

demonstrated that treatment of bean plants with glyphosate reduced phaseollin production in bean hypocotyls which resulted in the failure of bean plants to delimit the size of lesions caused by incompatible races of *Colletotrichum lindemuthianum.*

Glyphosate-induced predisposition of plant roots to disease was reported by Brammal and Higgins (1987). They showed that colonization of root tissues in tomato seedlings genetically resistant to *Fusarium oxysporum* f.sp. *radicis-lycopersici* occurred following exposure to a sublethal dose of glyphosate 24 h prior to inoculation. They observed that the glyphosate-induced colonization was associated with an inefficiency in incorporation of phenolic materials into the papillae and into modified cortical cell walls that would normally have formed as resistance responses to this pathogen. Recent research indicates that glyphosate did not suppress phytoalexin accumulation in roots of bean plants growing in a hydroponic system, but did significantly reduce lignification (Liu, 1995). Although this finding is not consistent with the results obtained by Holliday and Keen (1982) and Johal and Rahe (1984), its relevance to explain the glyphosate-induced disease predisposition in plants is supported by the following reports on the role of lignin in pathogenesis. Miller *et al.* (1966) showed that colonization of root cells by *Pythium* was limited to those cells that lack secondary cell-wall thickenings made up of lignin. McClure and Robbins (1942) also demonstrated that lignification of cell walls limited the advance of *P. splendens* in cucumber seedlings. The effect of glyphosate in reducing root lignification is not surprising, since the main target of glyphosate in plants, the shikimic acid pathway, is also the source of precursors for lignin production (Steinrucken and Amrhein, 1984).

Glyphosate synergistic interaction. In 1984, Johal and Rahe observed that bean seedlings treated with a certain dose of glyphosate died when grown in non-sterile soil, but survived in sterilized soil. They demonstrated clearly that plants died after glyphosate treatment due to fungal colonization of the roots, principally by the soilborne fungi *Pythium* and *Fusarium*. They called this plant-herbicide-fungus relationship glyphosate synergistic interaction or GSI.

Confirmation that soil microorganisms can alter the herbicidal action of glyphosate in plants came from the results of studies by Lévesque *et al.* (1992). They showed that seedlings of six different plant species were less sensitive to glyphosate when growing in heat-treated soil than in the corresponding natural soil. In autoclaved soil, the herbicidal effect of glyphosate was restored by addition of an aqeuous extract from natural soil but not if that extract was filtered (0.2 μm) or autoclaved. These findings clearly indicated the role of soil microorganisms in the lethal effect of glyphosate on various plants tested.

Results of studies conducted to date have shown that the main colonizers of glyphosate-treated seedlings are *Pythium* and *Fusarium* spp. (Johal and Rahe, 1984; Lévesque *et al.*, 1993a). Thus, the existence of GSI in plants is well documented, but basic information regarding the ecology of glyphosate synergistic fungi (GSF), and possible specificities between GSF and different plant species is still limited. Hence, a close examination of the role of soilborne microorganisms in GSI is needed. To achieve this goal, it is practical to concentrate research efforts on a single genus of soilborne fungus involved in GSI, to enable examination of the

distribution and host specificity of one glyphosate synergist in various soils, and the potential pathogenic effects on different plants.

Among the two genera of soilborne fungi that are involved in the GSI, *Pythium* appears to be the most promising and important candidate for extensive study. Lévesque *et al.* (1993a) showed that in bean and wheat seedlings grown in pots, colonization by *Pythium* spp. was detected at a higher frequency from the roots of glyphosate-treated seedlings than from the roots of untreated seedlings. *Pythium* is also one of the most studied soilborne fungus because several members of this genus are important plant pathogens (Hendrix and Campbell, 1973). Understanding the role of *Pythium* species in the GSI would be made easier if relevant information regarding the basic biology, activity and ecology of members of this genus in the soil are known, and this is the subject of Section 2.

Section 2. The Genus *Pythium*

In 1858, the genus *Pythium* was first established by Pringsheim based upon the descriptions of *P. monospermum* and *P. entophytum*, and was classified under the family Saprolegniaceae. Schroter in 1897 transferred *Pythium* to a new family, Pythiaceae, order Perenosporales, and class Oomycetes. Since then, its classification has remained unchanged (Domsch *et al.*, 1980). There were over 200 species described in old diagnostic keys (Middleton, 1943), while the most recent comprehensive classification keys include 87 species and two varieties (Vander Plaats-Niterink, 1981) and 125 species and four varieties (Dick, 1990), respectively.

Pythium spp. are widely distributed in nature. Their wide range of adaptation is indicated by their ability to cause diseases in many plants (Hendrix and Campbell, 1973), and in some cases in animals, such as fish (Scott and O'Brien, 1962), insects (Saunders *et al.*, 1988) and horses (Alfaro and Mendoza, 1990).

Biology and Life Cycle of *Pythium* in Soil:

Pythium spp. have hyaline, irregularly branched, coenocytic hyphae when young but as the mycelium matures, septa are produced to separate empty portions of hyphae and to delimit reproductive from vegetative structures. This fungus typically reproduces asexually by forming sporangia and sexually by oospores. Sexual reproduction takes place by formation of oogonia (female) and antheridia (male), and both structures are present in one individual in homothallic types or in two different individuals for heterothallic types. Fertilization occurs when an antheridium touches an oogonium, producing a thick-walled oospore (Van der Plaats-Niterink, 1981).

Asexual reproduction by zoospore formation from either sporangia or oospores occurs in some *Pythium* species. This method of reproduction is also a method of dispersal and is favored by high soil moisture conditions (Ayers and Lumsden, 1975). The process occurs under wet condition and starts by formation of a discharge tube from either a sporangium or an oospore. This is followed by protoplasmic streaming of the sporangium or oospore contents towards the thin-walled vesicle at the end of the tube where zoospore differentiation takes place. Agitation due to zoospore movement causes the vesicle wall to rupture, liberating the zoospores to swim away propelled by their two lateral cilia. Zoospores

swim towards the root tips of potential hosts and then encyst as spherical forms.

Germination Phase. Resting structures such as oospores, sporangia and encysted zoospores are stimulated to germinate in the soil by seed or root exudates (Nelson, 1987). Germination takes place by the production of germ tubes. If the soil environment is unfavorable, the germinating oospore, sporangia and zoospores may form another resting structure, or they may be lysed due to antagonistic microbial interactions (Hendrix and Campbell, 1973).

Infection phase. A germinated propagule forms an infection peg on the host surface and enters the host tissue directly through the cell wall or indirectly by intercellular penetration into the host tissues (Endo and Colt, 1974). The virulence of members of the species is largely determined by its ability to produce cell-wall-degrading enzymes (Dube and Prabakaran, 1989). Various pectolytic enzymes have been demonstrated in the following plant pathogenic species of *Pythium*: *P. aphanidermatum, P. debaryanum, P. dissotocum, P. irregulare, P. perniciosum, P. sylvaticum,* and *P. ultimum* (Singh and Singh, 1984). Cellulytic activity was also shown to be present in *P. aphanidermatum, P. debaryanum, P. graminicola, P. intermedium, P. irregulare, P. sclerotichum,* and *P. sylvaticum* (Deacon, 1979).

Fungal mycelium invades cortical tissues extensively both intra- and inter-cellularly (Nemec, 1971). Necrosis of root cells occurs even in advance of invading hyphae (Koike, 1971). *Pythium* may invade the stele tissues through those endodermal cells in which secondary thickenings

are not completed (Adegbola and Hagedorn, 1969). The pathogen may form resting structures such as sporangia or oospores on necrotic plant parts (Benard, 1994).

Saprophytic phase. *Pythium* may survive in the host tissues as mycelium, and may further colonize necrotic tissues saprophytically in the soil before forming oospores following exhaustion of nutrients or microbial antagonism (Hancock, 1981). Oospores present in soil may germinate in the vicinity of a non-colonized substrate and colonize it saprophytically before forming another batch of oospores (Agnihotri, 1984). *Pythium*-colonized residues in soil may be attacked by other microbes, resulting in disintegration of plant debris that contains *Pythium* propagules. This facilitates dissemination of inoculum in soil through tillage and other farming practices. The fungal propagules remain dormant in soil until they are stimulated to germinate and repeat an infection cycle (Burr and Stanghellini, 1973).

Techniques in *Pythium* Identification

The traditional method for identifying *Pythium* species is based on the presence or absence and characteristics of sexual and asexual reproductive structures and on colony morphology in certain culture media. Many difficulties are encountered in using morphological characterestics as a basis for identification of *Pythium* species since most of the species have similar structures. The diagnostic structures, when present, may display environmental variations and as a consequence, identification errors are common.

The problems associated with phenotypic variation are avoided if the *Pythium* genome is characterized directly. Recent advances in DNA

technology now permit the rapid and reliable characterization of the genome using restriction endonucleases. This technique provides a new tool for reliable identification of *Pythium* species. Martin and Kistler (1990) found that restriction endonuclease-digested mitochondrial DNA from 29 *Pythium* spp. showed distinctly different species-specific electrophoretic banding patterns. Chen (1992) used differences in the electrophoretic banding patterns of endonuclease-digested PCR-generated rDNA internal spacer (ITS) region to differentiate three heterothallic and five homothallic species of *Pythium.* DNA probes for sequences which are conserved within the genus *Pythium* but are species-specific have been developed for a limited number of species. Martin (1991) has constructed probes for *P. oligandrum* and *P. sylvaticum.* Lévesque *et al.,* (1994) have developed a probe for *P. ultimum,* and they are currently developing specific probes for other major species of *Pythium.*

Other techniques which have been used for *Pythium* species identification include differences in cellular proteins (Clare *et al.,* 1968; Adaskaveg, *et al.,* 1988) and isozymes (Chen *et al.,* 1991). However, problems in strict standardization of methods were found to be a major obstacle in obtaining reliable results from these tests. Hence, these techniques are considered less effective than DNA analysis.

Despite the inherent difficulty associated with using morphological structures for identifying *Pythium,* it remains the principal method used for species identification today. However, DNA pattern differences among various similar isolates are increasingly used to confirm identifications based on morphology of reproductive structures. In the near future, molecular techniques will undoubtedly replace or complement the

traditional method of *Pythium* identification, and will provide a sound basis for accurate identification of isolates which do not produce sexual structures.

Symptoms of *Pythium* Diseases in Plants:

Damping-off. The damping-off symptoms caused by *Pythium* vary with age and stage of development of infected plants. When seeds of susceptible plants are sown in infested soils and are infected by *Pythium*, they fail to germinate, become soft and mushy, then turn brown, shrink, and finally disintegrate. Seed infections taking place in soil are not observed, and the only manifestation of disease is poor emergence. Poor stands are also the result of infection of seedlings by *Pythium* after the seeds have germinated but before the seedlings have emerged above the soil surface. Tissues of young seedlings can be attacked at any point. The site of infection initially appears as a slightly darkened, water-soaked spot. The infected area enlarges rapidly, the invaded cells collapse, and the seedling is overrun by the fungus and dies shortly after initiation of the infection. In cases were infection of seedlings takes place before seedling emergence, the disease is called pre-emergence damping-off (Agrios, 1988).

Seedlings that have already emerged are usually attacked in the roots and sometimes at or below the soil surface. The succulent tissues of seedlings are easily penetrated by *Pythium*, which invades and kills the cells rapidly. The invaded areas become water-soaked and discolored, and the cells soon collapse. This stage of infection causes the basal part of the seedling stem to be much thinner and softer than the above, as yet uninvaded, parts. Owing to loss of firmness and support in the invaded

portion, the seedling falls over. The fungus continues to invade the seedling after it has fallen to the ground, and the seedling quickly withers and dies. This phase of the disease is called post-emergence damping-off (Agrios, 1988).

Infection of mature plants and soft rot of succulent tissues. When older plants are attacked by *Pythium*, they usually show only small lesions on the stem. If lesions are sufficiently large or numerous, they can girdle the plant and cause stunting or death (Kraft and Burke, 1971). More commonly, infection of older plants is limited to rootlets. Infected roots of mature plants are damaged and frequently killed by the fungus, resulting in stunting, wilting, and eventual death of the shoots (Roncadori and McCarter, 1971; Mircetich, 1971).

Soft fleshy organs of some vegetables, such as cucurbit fruits, green beans, potatoes, and cabbage heads are sometimes infected by *Pythium* during extended wet periods in the field, in storage, and in transit (Singh and Singh, 1984). Such infections result in a cottony fungus growth on the surface of the fleshy organ, while the interior turns into a soft, watery, rotten mass called "leak" (Agrios, 1988).

Factors Affecting *Pythium* Survival in Soil

The soil environment is comprised of soil factors, plant factors, and soil-dwelling organisms including *Pythium*. Since *Pythium* is only one of many components in a complex soil ecosystem, other factors of the soil environment will affect its activity both directly and indirectly. Many of the positive and negative impacts of these factors on the survival of *Pythium* in soil have been documented.

Pythium spp. found in soil range from a saprophytes to facultative parasites of plants. Physical soil factors such as pore spaces, water, aeration, temperature, and chemical factors, such as organic matter content and pH, directly affect the prevailing microenvironment in the soil, and as a consequence can indirectly influence the saprophytic and parasitic activities of resident *Pythium* populations.

Of these factors, presence of virgin organic matter, soil moisture, soil aeration and soil temperature are the most important in saprophytic growth of *Pythium* spp. in soil. *Pythium* generally needs an abundant supply of virgin organic substrate for saprophytic growth. The saprophytic phase occurs mainly under conditions where other microorganisms are not present or are greatly reduced in activity because most *Pythium* spp. do not compete well for substrates already colonized by a well-established microflora (Barton, 1961). Thus, the saprophytic phase of growth in an undisturbed soil, where new organic substrates are infrequently available, is usually shortlived and greatly affected by other soil microorganisms (Hendrix and Campbell, 1973).

Griffin (1963) found that *P. ultimum* grew well saprophytically under conditions where soils were saturated with water. Gardner and Hendrix (1972) found that elevated levels of carbon dioxide in soil favored saprophytic activity of *P. irregulare* and *P. vexans*. The ability of *Pythium* to tolerate high moisture and poor gas exchage give it an ecological advantage for saprophytic growth because these same conditions are unfavorable for other microbes, including *Pythium* antagonists (Mircetich, 1971).

The effect of temperature on saprophytic activity was directly correlated with the optimum temperature requirements in culture. When

field soil was stored at various temperatures, populations of *Pythium* species with high temperature optima increased during high temperature storage, while species with lower temperature optima decreased (Golden *et al.*, 1972).

Soil moisture, aeration, temperature, pH and mineral nutrient availability affect the *Pythium* parasitic phase in soil both directly and indirectly. As in the saprophytic phase, soil moisture is an important limiting factor for parasitic activity of *Pythium* in soil. The effect of soil moisture on *Pythium* is substantially species dependent. Some species, e.g. *P. graminicola* and *P. arrhenomanes*, have more tolerance to low moisture than other species, such as *P. aphanidermatum* and *P. butleri* (Kouyeas, 1964). The pathogenic stage of zoospore-producing species such as *P. vexans* is most influenced by high moisture conditions (Biesbrock and Hendrix, 1970), which increase production of inoculum by favoring zoospore formation from a sporangium over direct germination. If a sporangium germinates by a germ tube, only one infective propagule develops, but when germination by zoospores takes place, many individuals may arise frcm a single sporangium. In addition, zoospore formation from a single sporangium of some *Pythium* species can be repeated as many as six times (Agnihotri, 1984). The effects of soil moisture and soil aeration on *Pythium* parasitic activity in soil can be interrelated because the portion of soil pore space not occupied by water molecules is filled with gas. Saturated soil conditions result in poor soil aeration, which affect *Pythium* indirectly by reducing plant vigor and increasing root exudation, which stimulate germination of *Pythium* dormant propagules (Nelson, 1991).

The effect of soil temperature on the *Pythium* parasitism can occur in two ways: directly on pathogen and indirectly on host. *Pythium* species have different temperature optima in culture, and this relates to differential temperature responses among species associated with infection and parasitic activity. For example, Gold and Stanghellini (1985) found that severe damping off of hydroponic-grown spinach caused by *P. aphanidermatum* occurred only during the summer months, when temperatures ranged from 21-27°C, while damping-off caused by *P. dissotocum* occurred during cold winter months, with average temperatures of about 17°C. Similarly, seasonal differences in root colonization of alfalfa were evident, with *P. ultimum* predominant in the summer, and *P. irregulare* in the winter and early spring (Hancock and Grimes, 1990). Extreme soil temperatures can also affect *Pythium* indirectly by predisposing a host plant to infection, e.g., by inducing root exudation, thereby making the plant more susceptible to *Pythium* attack (Kraft and E_win, 1967).

Soil pH can affect *Pythium* growth directly or indirectly by mediating changes in plant growth and microbial activity that will either enhance or inhibit pathogen growth. Plants growing in soils with unfavorable pH are susceptible to *Pythium* infection due to poor health (Hendrix and Campbell, 1973). Studies done by Qian and Johnson (1987) indicated that raising soil pH from 4.3 to 6.1 reduced *P. ultimum* oospore survival due to enhanced antagonist activity in the soil. It is a common farming practice to increase soil pH by applying lime to increase productivity. Liming also is used to reduce toxicity of excess micronutrients in the soil to plants. Calcium from the liming material ($CaCO_3$, $Ca(OH)_2$ and $CaSO_4$) enhanced the suppressiveness of soils to

P. splendens. The reasons for this phenomenon are not fully known. Kao and Ko (1986) suggested that soil antagonists of *Pythium* became more active because they could readily obtain nutrients from the environment in the presence of high calcium. Calcium amendment can also increase root growth and the over-all growth rate of plants, which can shorten the susceptible period and result in reduced infection. It is also possible that increased calcium uptake by plants reduces the susceptibility of plant cell walls to lysis by enzymes produced by *Pythium* during infection (Punja, 1989).

Nitrogen fertilization is known to enhance or suppress *Pythium* in the soil. High nitrogen may increase susceptibility to *Pythium* infection by prolonging succulent host growth and juvenility. However, when applied as urea, a negative effect on *Pythium* was observed in the form of reduced inoculum in soil. This was due to toxicity of gases released from urea when it was degraded by soil microbes into ammonia and carbon dioxide (Chun and Lockwood, 1985).

Microbial Interactions Affecting *Pythium* in Soil

Bacteria, fungi and other small soil-dwelling organisms such as protozoans, nematodes, and arthropods, can compete with *Pythium* for substrates, or use *Pythium* as a substrate in soil. The mechanisms of microbial interactions involving *Pythium* in soil vary considerably for every microorganism concerned, ranging from competition and antagonistic biochemical interactions against *Pythium*, to actual parasitism and predation of *Pythium* hyphae (Curl and Harper, 1990).

Pythium is a naturally poor competitor for space and is usually unsuccessful in invading a pre-colonized substrate (Marx, 1972). This

was aptly demonstrated by Elad and Chet (1987), who protected cucumber plants from *Pythium* damping-off by coating the seeds with antagonist bacteria (*Pseudomonas putida* and *P. cepacia*) before planting.

Pythium spp. are known to be affected by antibiotic metabolites produced by bacteria and fungi. These metabolites may be specific or non-specific lytic agents, enzymes, volatiles or other toxic compounds. For example, certain fluorescent pseudomonads such as *P. fluorescens*, strain Pf-5, produce pyoluteorin, which is effective against *P. ultimum* damping-off in cotton (Howell and Stipanovic, 1980). Another strain of *P. fluorescens*, 2-79, isolated originally from a suppressive soil also produced an antibiotic identified as phenazine-1-carboxylic acid, which is inhibitory to a number of fungi, including *Pythium aristosporum* (Gurusiddaiah *et al.*, 1986). A fungal organism such as *Gliocladium virens*, may produce an antibiotic metabolite called gliovirin which is active against *P. ultimum* damping-off of cotton (Howell and Stipanovic, 1983).

Numerous soil bacteria have been shown to be capable of lysing *Pythium* hyphae and propagules in soil. *Enterobacter cloacae* controlled *Pythium* in peas, sugar beets, and cucumber by degrading the fungus mycelium (Hadar *et al.*, 1983). *Pseudomonas cepacia*, strain AMMD, inhibited mycelial growth and lysed zoospores of *Pythium aphanidermatum* (Parke, 1990).

Pythium are known to be parasitized by other fungi, ranging from chytridiomycetes to basidiomycetes. Some mycoparasites seem to specialize on *Pythium*, such as *Woronina pythii*, a plasmodiophoromycete which can parasitize 15 species of *Pythium* (Dylewski and Miller, 1983). Some non-plant pathogenic *Pythium* can parasitize pathogenic *Pythium*

and other fungi. For example, *P. nunn* can displace *P. ultimum* from a previously occupied substrate by parasitizing its hyphae (Paulitz and Baker, 1988). *P. nunn* also formed appressoria-like structures and parasitized *P. aphanidermatum, Rhizoctonia solani, Phythophthora cinnamomi* and *P. parasitica* (Lifshitz *et al.*, 1984).

Pythium are also preyed upon by small soil-dwelling animals like protozoans, arthropods, soil mites and nematodes. Their impact on *Pythium* populations are not easily discernable, but if the various *Pythium* predator populations were high enough, large numbers of *Pythium* propagules could be affected.

In summary, the presence of *Pythium* in soil is naturally influenced by interactions of soil, plants and soil-dwelling microorganisms, such that an equilibrium is achieved. Thus, in an undisturbed soil ecosystem, the *Pythium* population could be naturally regulated by predators and parasites. Man-made changes imposed on the natural ecosystem, as in modern agricultural practices, may shift the natural equilibrium in favor of *Pythium*, resulting in enhanced saprophytic and parasitic activity in soil. As a result, an increased frequency of soilborne seedling diseases has been observed to occur in agricultural areas from various countries where weeds were previously killed with glyphosate before planting of crops (Davies and Davies, 1981; Tesar, 1984; Blowes, 1987; Roget *et al.*, 1987; and Smiley *et al.*, 1992). It was speculated that the observed cases of increased seedling disease in glyphosate-treated areas may have been the result of the phenomenon called glyphosate synergistic interaction as described in Section 1. Since very little is known about the basic mechanisms of GSI, a focused examination of the phenomenon is necessary to assess its role in enhancing *Pythium* diseases in the field.

In this thesis, the role of *Pythium* spp. as glyphosate synergistic fungi were examined with the following objectives:

a) to determine the occurrence and distribution of the glyphosate synergistic *Pythium* in various soils;

b) to determine the host specificity of *Pythium* spp. as glyphosate synergists; and

c) to examine the effect of glyphosate-treated plants on the population dynamics of *Pythium* in natural soil and in *Pythium*-inoculated soil.

Chapter II

ISOLATION, IDENTIFICATION AND TESTING OF *PYTHIUM* SPECIES AS GLYPHOSATE SYNERGISTS

INTRODUCTION

Previous research has shown that the effect of the herbicide glyphosate on plants can be enhanced by certain soilborne fungi that act as glyphosate synergists (Johal and Rahe, 1984; Rahe *et al.*, 1990; Lévesque and Rahe, 1992). *Pythium* species appear to be the predominant root colonizers of glyphosate-treated seedlings (Lévesque *et al.*, 1993a), and their involvement as glyphosate synergists is of particular interest since many species of this genus are economically important soilborne plant pathogens.

Glyphosate-induced predisposition of plants to root infection by facultative parasites such as *Pythium* is hypothesized to be the outcome of glyphosate's mode of action in plants. Glyphosate blocks the synthesis of phenylalanine-derived phenols by its inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Amrhein *et al.*, 1980), thereby reducing the production of phenolic compounds such as lignin precursors and some classes of phytoalexins that may be involved in resistance of plants to root diseases (Brammall and Higgins, 1987).

There is currently no information on the *Pythium* species that function as glyphosate synergists and on their distribution in different soils. To address this need, a study was conducted: i) to characterize the different *Pythium* species involved in the glyphosate synergistic interaction (GSI) and their current distribution in diverse soils; ii) to assess the degrees of enhancement of glyphosate efficacy by different

glyphosate synergistic *Pythium* (GSP) isolates on bean seedlings; and iii) to determine the virulence of proven GSP isolates on bean seedlings not treated with glyphosate.

MATERIALS AND METHODS

Isolation of Putative GSP

Three soils from the Okanagan Valley near Summerland, B.C., and two from the Fraser Valley near Aldergrove, B.C., Canada were used. Two of the Summerland soils were collected from orchards with known histories of apple crown rot and apple replant diseases, and the remaining soil sample was from an area without a known history of agricultural cropping. These soils were designated as crown rot soil (CRS), apple replant soil (ARS), and virgin soil (VS), respectively. The Fraser Valley soils were obtained from sites that were used for vegetable and apple tree nursery stock production. The soils were designated as muck soil (MS) and mineral loam soil (LS) based on their structural characteristics.

All soils were collected from the field sites within 8 weeks before the start of the experiments, using tools and containers disinfected with 70% EtOH. The collected soils were sieved (5-mm mesh) to remove stones and large organic debris, and stored in plastic containers until needed.

Seeds of bush bean (*Phaseolus vulgaris* L. cv. Topcrop) were surface disinfected by soaking in 1% NaOCl solution for 5 min and washing in three changes of distilled water before planting. An individual bean seed was planted in each of 25 polystyrene cups, each cup was filled with 150 g of field soil, five from each soil source. The cups were

placed in a growth chamber set at 16 h daily photoperiod, 25:19°C day:night temperatures, and were watered twice a week with distilled water to keep the soil normally moist. The light intensity at plant height was 230 μmol m^{-2} s^{-1} and the prevailing daytime RH was 50%.

Glyphosate was applied at 2.5 μg a.i. per plant when the seedlings were 2 weeks old. The dose was two, 1.25 μL droplets of diluted commercial herbicide, Roundup® (Monsanto Corp., St. Louis, Mo.), on each primary leaf. The plants were harvested at 0, 2, 4, and 6 days after the glyphosate treatment. The roots were then washed under running water and plated on *Pythium*-selective medium (Mircetich, 1971) using the plating technique developed by Lévesque, *et al.* (1993a). Fungal colonies growing from the plated roots were isolated into pure cultures and maintained on potato dextrose agar (PDA) under mineral oil or in autoclaved segments of grass leaf in sterile water at 22°C. Stored isolates were revived by transferring small portions of the cultures onto fresh PDA.

Identification of *Pythium* isolates:

Colony morphology, growth rate, and reproductive structures. Sixty-five *Pythium* isolates were inoculated separately onto solidified 16 ml PDA in 100x15 mm petri plates by transferring 1 cm diameter plugs to the centers of the plates. The inoculated plates were incubated in darkness at 22°C. Six replicate plates were used for each isolate tested. Radial growth was estimated by measuring colony diameters in two dimensions 24 h after inoculation.

The *Pythium* isolates were induced to form reproductive structures using various methods as described by Waterhouse (1968). The

morphology of reproductive structures, growth rates, and colony morphology were used to identify the *Pythium* isolates to the species level, using keys developed by Waterhouse (1968) and Van der Plaats-Niterink (1981). Fifteen representative cultures of identified *Pythium* isolates were sent to Dr. Donald J.S. Barr, Biosystematics Research Institute, Ottawa, ON, Canada for confirmation of the identifications.

Differentiation of *Pythium* isolates by Restriction Fragment Length Polymorphisms (RFLP) of total DNA. Each of the 65 isolates of *Pythium* was revived from the stock cultures by tranferring a small portion of the mycelia onto fresh PDA, and incubating at 22-24°C in the dark for 5-6 days. Each isolate was separately inoculated as five 0.5 cm diam plugs in a 250 mL Erlenmeyer flask containing sterile 100 mL V8 juice broth. The flasks were incubated in darkness at 22-24°C for 7-8 days; the mycelial mats were then harvested, and washed with cold (5°C) 0.1 M NaCl solution by vacuum filtration on a ceramic funnel lined with Whatman No. 1 filter paper.

A modified version of the procedure described by Curran *et al.* (1985) was used for DNA extraction. The washed mycelium was mixed with extraction buffer (0.1 M Tris-base, 0.05 M Na_2EDTA, 0.2 M NaCl, 1.0% SDS, pH 8.0) at 4 mL per g of mycelium, before freezing in liquid nitrogen. The frozen mycelia were held in a -20°C freezer until the DNA was extracted. The frozen mycelium was ground to a fine powder with a mortar and pestle and after lysis at 22-24°C, and transferred to a centrifuge tube. A half volume of phenol (Bethesda Research Laboratories [BRL], Inc) was added to the lysed mycelium and mixed by gentle inversion of the tube, followed by centrifugation at 3500 rpm for 15 min.

The upper aqueous phase was removed to a new centrifuge tube and DNA extraction by phenol of the same lysed mycelium was repeated two more times. The collected aqueous phase was then treated with chloroform-isoamyl alcohol (24:1, v:v) and the aqueous phase was transferred into a new centifuge tube. The treatment with chloroform-isoamyl alcohol was made three times. The collected DNA from the aqueous phase was precipitated by addition of two volumes of 95% EtOH and then centrifuged for 5 min. The 95% EtOH was slowly decanted and the DNA was reprecipitated using 70% EtOH, and then briefly centrifuged to accumulate the DNA in the bottom of the tube. The 70% EtOH was discarded and the DNA was resuspended in 0.5 mL of TE buffer (10mM Tris-HCL, 1 mM Na_2EDTA, pH 8.0). The suspension of DNA in TE buffer was treated with 5 mg RNAse (Sigma) and incubated at 37°C for 30 min. Further purification was achieved by adding 4 M NaCl to bring the concentration up to 0.2 M and repeated precipitation with 95% and 70% ethanol as described above. The DNA pellet obtained after the addition of 70% ethanol was dried under vacuum and then dissolved in 50-100 µL of TE buffer and stored at 4°C.

Hind III and *EcoR* I restriction enzymes were used to digest the total DNA. Approximately 1 µg total DNA from each isolate was incubated with either *Hind* III or *EcoR* I at 37°C for 2 h. The digests were then mixed with loading buffer (10X =15% Ficoll 400, TBE 8X, 0.25% bromophenol blue, and 0.25% xylene cynole) and loaded into wells made in the gel. The digests of isolates with similar colony characteristics were electrophoresed in separate lanes in the same gel to facilitate comparison among isolates. Electrophoresis was done at 0.5 V cm^{-1} in 0.7% agarose-ethidium bromide gel submerged in a 1X TBE buffer (89 mM Tris-HCL,

2.5 mM Na$_2$EDTA, 89 mM boric acid, pH 8.3). A 1 Kb DNA ladder (BRL) was used as a size marker. The gels were photographed over a 300 nm UV light source to record the banding patterns. The images of the banding patterns of the representative RFLP types from each RFLP group were transferred from the photo negatives onto paper by tracing the DNA bands.

Statistical analysis of growth rates and RFLP data

Variance Components Estimation Procedure (SAS Institute Inc., 1985) was done on the growth rate data to estimate the variation due to species, RFLP class within species, isolates within RFLP class, and plates within isolates. All the factors were treated as random.

Pythium isolates as glyphosate synergists

The potential of *Pythium* isolates to enhance the herbicidal efficacy of glyphosate was quantified by comparing LD$_{50}$ values estimated on bean seedlings (cv. Topcrop) grown under controlled conditions in the absence or presence of *Pythium*. The isolates were chosen randomly from each of the different *Pythium* RFLP types and were obtained from roots of glyphosate-treated bean seedlings grown in five different soils.

Components of the method used for estimating LD$_{50}$ values were selected based on preliminary tests which compared different plant growing media, concentration of *Pythium* inoculum, methods of inoculation and the use of plants growing individually in polystyrene cups or in rows in plastic trays. The method chosen was to plant surface sterilized bean seeds in plastic trays (54x28x7 cm) filled with 3.5 kg autoclaved sandy loam soil. Twenty seeds were planted in each of six

equally-spaced rows, oriented across the width of each tray. The plants were kept in a growth room under conditions already described. The soil was kept moist by addition of distilled water as needed. The plants were watered with a dilute solution of 20-20-20 fertilizer one week after planting.

For inoculum production, individual *Pythium* isolates were grown in stationary culture in V8 cholesterol broth (Ayers and Lumsden, 1975) for 8 days. The mycelium was filtered and rinsed with sterile distilled water in a Büchner funnel lined with filter paper. The mycelial mat was cut aseptically into 1x1 cm pieces with a scalpel. The mycelium pieces were suspended in 0.08 % sterile water agar (WA) at 1 g per 100 mL, then macerated in a Sorvall blender for 20 sec. Inoculation was done 1 day before glyphosate treatment by drenching 25 mL of the blended inoculum suspension between each row of seedlings when the seedlings had two fully expanded primary leaves (2 weeks after planting). Glyphosate treatments were made 1 day later, using 2 µL droplets applied to the stem at the cotyledonary node. Different glyphosate doses (0-1200 µg per plant) were assigned at random to each row of plants, but all plants in a row received the same dose. The numbers of plants killed by each dose were recorded 4 weeks after glyphosate treatment. One tray was used for each *Pythium* isolate per experiment and the experiment was repeated at least twice.

Data for the proportion of dead plants at different doses of glyphosate from each experiment were analyzed by stepwise logistic regression using the GLIM statistical program (Baker and Nelder, 1987). The full logistic regression model was $\pi = \dfrac{e^{\alpha_i + \beta_i x}}{1 + e^{\alpha_i + \beta_i x}}$, an S-shaped curve that can be linearized by using the logit transfomation,

$\ln\left(\dfrac{\pi}{1-\pi}\right) = \alpha_i + \beta_i \chi$. In this model, π is the proportion of dead plants, X = ln (glyphosate dose + 1) and the intercept and the slope for the ith isolate are α and β, respectively. LD_{50} values were estimated by setting the regression expression for logit π in the fitted regression model equal to zero $(0 = \alpha + \beta X_{LD_{50}})$, i.e. $X_{LD_{50}} = -\alpha/\beta$ (Rahe *et al.*, 1990). Standard errors were calculated for the estimated X_{50} values using the propagation of errors formula for approximate variance of a transformed variable (Rice, 1988). The resulting untransformed LD_{50} values and their upper and lower asymetrical confidence limits (+ or - S.E.) were obtained by applying the exponential function $LD_{50} = \exp(X_{50}) - 1$ to the symmetrical upper and lower limits for ln (glyphosate dose + 1). Glyphosate LD_{50} values for plants inoculated with different *Pythium* isolates were compared with the control using Bonferroni's t-test (Neter *et al.*, 1990).

Pathogenicity of *Pythium* Isolates

The ability of different *Pythium* isolates to cause disease on germinating bean seeds was assessed by growing individual isolates for 5 days on PDA in 100x15 mm Petri dishes. The colony was then covered with sterilized peat-based potting mix (Metro-mix™ grade 290, W.R. Grace and Co. of Canada Ltd., Ajax, Ontario) to a depth of 5-6 mm. Twenty surface-disinfested bean seeds (cv. Topcrop) were put on the surface of the Metro-mix™ and additional Metro-mix™ was added to barely cover the seeds. Approximately 20 mL of sterilized distilled water was added and the plates were placed in individual plastic bags and placed into the same growth chamber used to grow plants for 7 days, after which the numbers of ungerminated seeds, and dead and live

seedlings in each Petri plate were recorded. Three replicate plates were used for each *Pythium* isolate.

The potential of isolates to cause disease on emerged bean seedlings was assessed in two ways. In one test, 25 mL of macerated mycelium was drenched at the base of 2-week-old bean plants growing in 500 mL sterilized Metro-mix™ contained in 15 cm diameter pots. One pot containing 20 seedlings was used for each isolate and the experiment was replicated three times. The Metro-mix™ was kept moist by watering with 100 mL distilled water every second day. The pots were placed in a controlled environment chamber set at the conditions stated earlier. Two weeks after inoculation, the numbers of dead and living plants were recorded. The second method of pathogenicity evaluation of the different *Pythium* isolates to cause post-emergence damping-off on 2-week-old bean plants was determined using the glyphosate-untreated plants assigned as controls in the GSI tests conducted. The number of dead plants was counted 4 weeks after inoculation. ANOVA was performed on arcsine transformed data using the SAS Statistical Package (1985).

RESULTS

Identification of isolates

A total of 65 *Pythium* isolates was collected from the roots of glyphosate-treated bean seedlings grown in five different soils. Six species were represented among the 65 *Pythium* isolates as identified by morphology of reproductive structures and colony characteristics. Almost all of the isolates produced cottony aerial mycelial growth on PDA

(Figure 1). Some isolates of *Pythium* 'G' group, *Pythium* 'HS' group and all isolates of *P. irregulare* had distinctly large rosette patterns. *P. coloratum* had appressed colony growth on PDA with a distinct smaller rosette pattern than that of *Pythium* 'G' and 'HS' groups and *P. irregulare* (Figure 1). The commonly observed morphological features of isolates of *P. ultimum*, *P. sylvaticum*, *P. irregulare* and *Pythium* 'HS' and 'G' groups are shown in Figure 2. Using a grass-blade culture technique (Waterhouse, 1968) at room temperature (22-24°C), some isolates of *P. ultimum* and all isolates of *Pythium* 'G' group readily released zoospores from globose sporangia-like structures (Figure 3) while *P. coloratum* released its zoospores from digitate, slightly inflated zoosporangia through long filamentous discharge tubes (Figure 3). None of the other *Pythium* isolates was observed to produce zoospores in response to any of the induction methods and temperature treatments used.

The species of *Pythium* and numbers of isolates obtained from the five soils are presented in Table 1. The highest number of isolates was obtained from MS (20) followed by CRS (17), LS (14), and ARS (12). Only two isolates were obtained from VS. *P. ultimum* and *Pythium* 'G' group were present and frequently isolated from ARS, CRS, LS, and MS. Other species, such as *P. irregulare*, *P. sylvaticum*, *P. coloratum*, and *Pythium* 'HS' group were sporadically present.

DNA Analysis

Fourteen and 15 different RFLP patterns were observed among the 65 *Pythium* isolates when the total DNA of each isolate was digested with
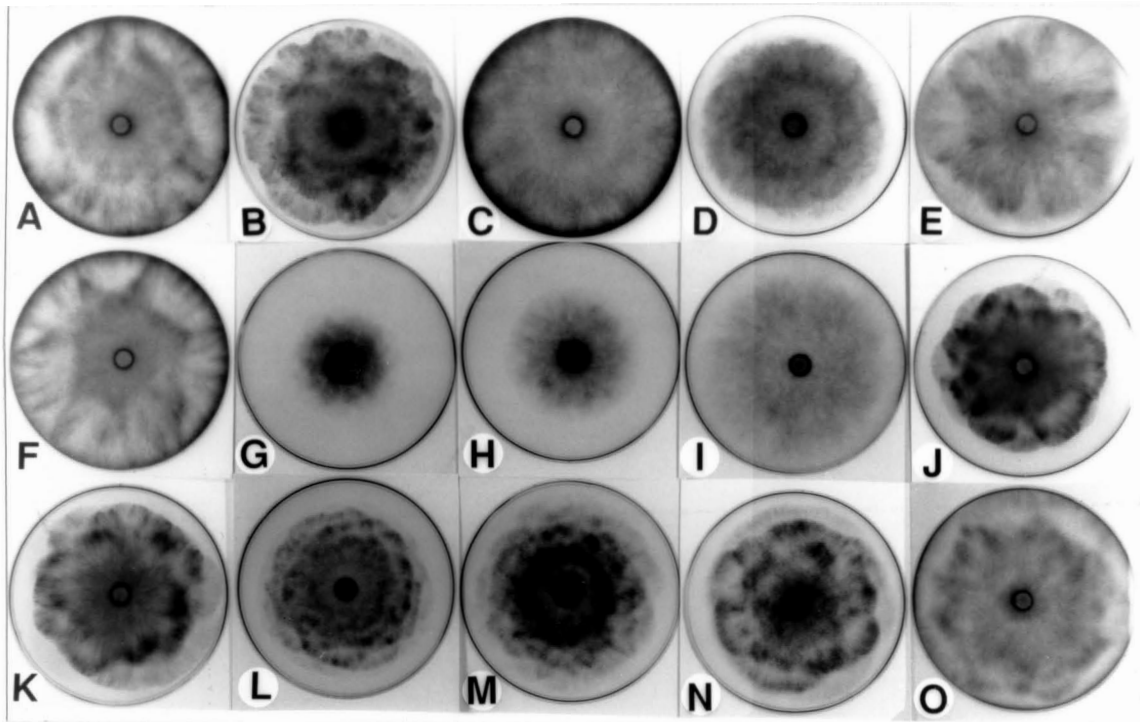
Figure 1. Colony characteristics of 15 representative *Pythium* isolates. *P. ultimum* (A and C), *Pythium* 'G' group (B), *P. sylvaticum* (D, E, F, G, H, and I) ; *P. irregulare* (J and K); *P. coloratum* (L and M); and *Pythium* 'HS' group (N and O).
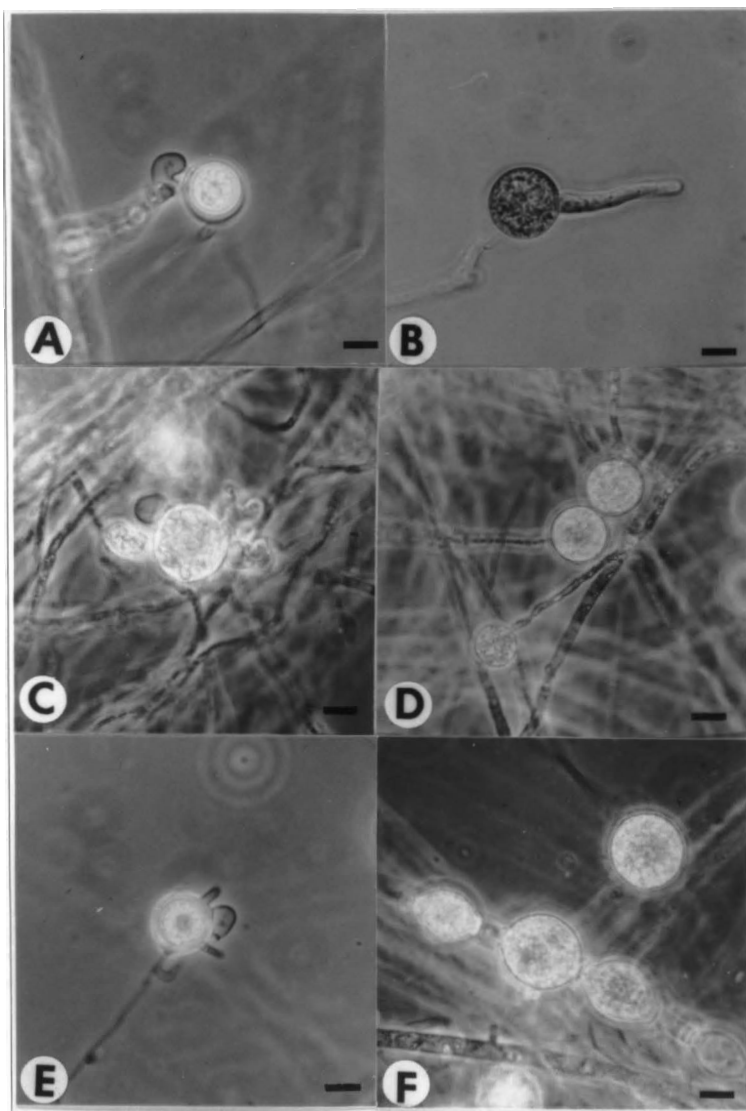
Figure 2. A) *Pythium ultimum* antheridium and oogonium, B) *P. ultimum* germinating sporagium, C) Antheridia and oogonium of compatible *P. sylvaticum*, D) *Pythium* 'G' group globose type sporangia, E) *P. irregulare* antheridium and oogonium, and F) *Pythium* 'HS' group hyphal swelling structures. Magnification A,C,D,E and F bar=0.2mm, B bar=0.02mm.
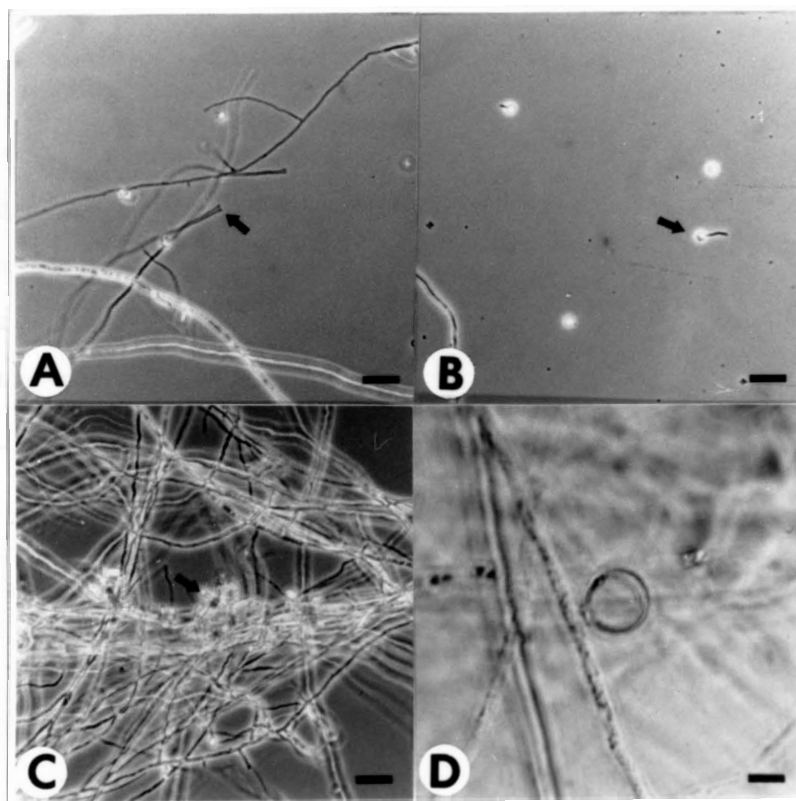
Figure 3. A) *Pythium coloratum* long filamentous zoospore discharge tube (arrow) B) *P. coloratum* germinating (arrow) and encysted zoospores, C) *P. coloratum* sporangia (arrow), D) An empty globose zoosporangium, the type seen in *P. ultimum* and *Pythium* 'G' group. Magnification A, B and C bar=0.2mm; D bar=0.02mm.

Table 1. Numbers of isolates of different species* and groups of *Pythium* obtained from roots of glyphosate-treated bean seedlings grown in five different soils.[α]

Pythium species and groups	Summerland soils			Fraser Valley soils		
	VS	ARS	CRS	LS	MS	Total
P. 'G' group	-	1	3	3	4	11
P. ultimum	-	4	7	9	6	26
P. irregulare	-	7	-	1	-	8
P. sylvaticum	2	-	6	1	5	14
P. coloratum	-	-	-	-	3	3
P. 'HS' group	-	-	1	-	2	3
Total:	2	12	17	14	20	65

[α] VS = virgin soil; ARS = replant soil; CRS = crown rot soil; LS = loam soil; and MS = muck soil.

* Identification of the species based on morphological characteristics of the isolates when grown on PDA and on grass-leaf cultures.

Hind III and *EcoR* I restriction enzymes, respectively (Figure 4A and 4B). Two types of banding patterns were noted in the *Hind* III digests of DNA of the *P. ultimum* isolates. The corresponding isolates were designated *P. ultimum* RFLP types 1 and 2. *Hind* III-digested DNA of all isolates of *Pythium* 'G' group had an identical DNA pattern with that of *P. ultimum* type 1. This similarity resulted in placing the *Pythium* 'G' group into the *P. ultimum* RFLP type 1 group. Six types of banding patterns were observed among isolates of *P. sylvaticum*, whereas isolates of *P. irregulare*, *P. coloratum* and *Pythium* 'HS' group each yielded only two patterns. These isolates were subsequently differentiated and designated as *P. sylvaticum* RFLP types 1,2,3,4,5, and 6; *P. irregulare* RFLP types 1 and 2, *P. coloratum* RFLP types 1 and 2; and *Pythium* 'HS' RFLP types 1 and 2.

Groupings of some isolates were shown to be identical with those derived from *Hind* III-digested DNA, by using *EcoR* I digests of total DNA of these isolates. However, more detailed information on isolates belonging in *P. ultimum* RFLP type 1 was derived by using *EcoR* I, since isolates originally placed under this RFLP type based on *Hind* III-digested DNA revealed two types of DNA patterns. As a result, *P. ultimum* RFLP type 1 was divided into two subgroups called *P. ultimum* type 1a and *P. ultimum* type 1b. Of the 26 isolates morphologically identified as *P. ultimum*, 19 had the RFLP type 1a pattern, 6 had RFLP type 1b, one had RFLP type 2. Out of 11 isolates identified as *Pythium* 'G' group, 7 and 4 isolates were of RFLP type 1a and RFLP type 1b pattern, respectively. The numbers of isolates occurring within each RFLP group are shown in Table 2.

A)
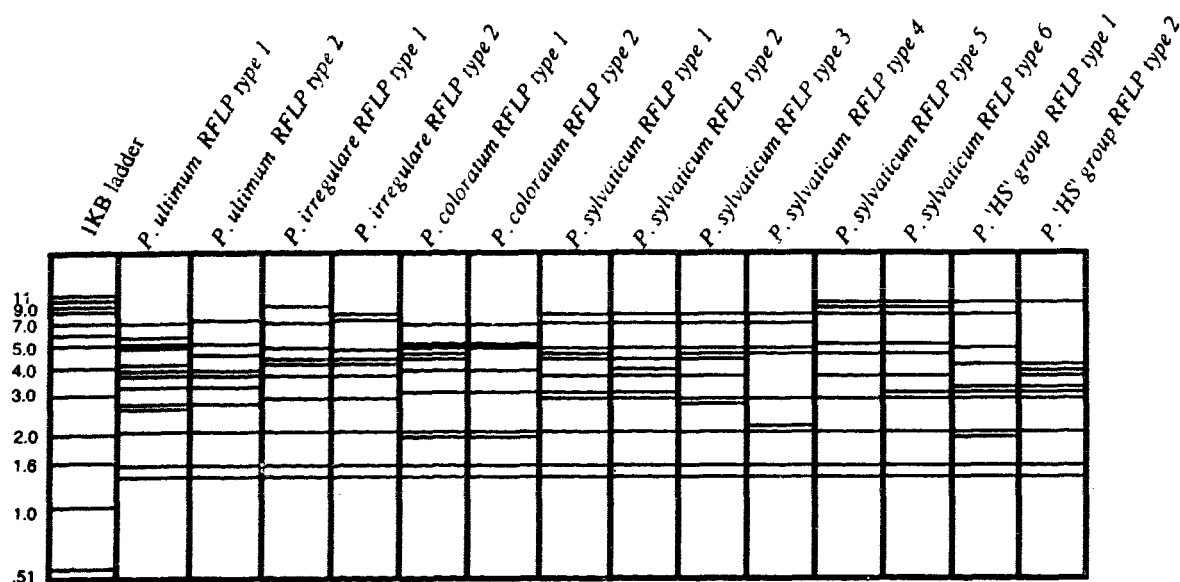
B)
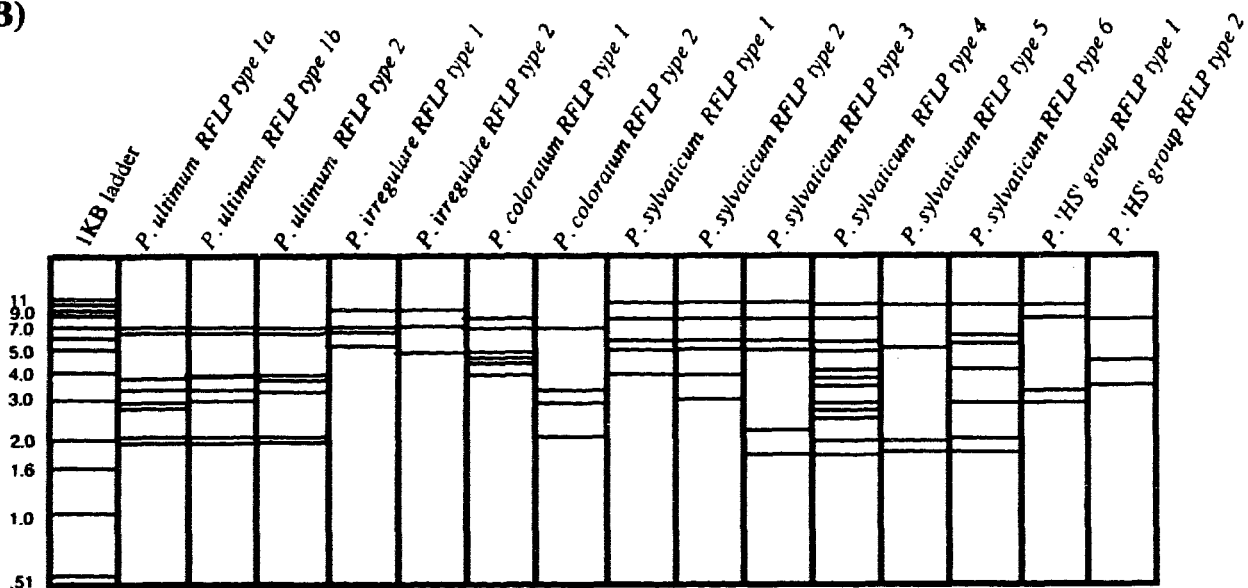
Figure 4. RFLP patterns of A) *Hind* III- and B) *EcoR* I-digested DNA of
Pythium isolates obtained from roots of glyphosate-treated
bean seedlings grown in five different soils.

Table 2. The relationship between growth rates of *Pythium* isolates in axenic culture and the number of days after treatment of bean plants with glyphosate at which those *Pythium* isolates were obtained from the roots.

Pythium RFLP type	Number of isolates within RFLP group	Colony diameter on PDA after 24 h (mm) $^\alpha$	Days after glyphosate treatment $^\beta$ 0 2 4 6			
P. ultimum 1a	25	42.4 a	−	+	+	+
P. ultimum 1b	11	42.5 a	−	+	+	+
P. sylvaticum 2	2	41.1 a	−	+	+	+
P. irregulare 1	7	39.9 ab	−	+	+	+
P. sylvaticum 3	2	39.8 ab	−	−	+	+
P. ultimum 2	1	37.5 bc	−	+	+	+
P. sylvaticum 1	3	37.4 bc	−	−	+	+
P. 'HS' 2	2	34.4 cd	−	+	+	+
P. 'HS' 1	1	32.1 de	−	+	+	+
P. irregulare 2	1	30.5 ef	−	+	+	+
P. sylvaticum 5	1	28.7 ef	−	−	−	+
P. coloratum 1	2	28.7 ef	−	−	−	+
P. coloratum 2	1	27.7 fg	−	−	−	+
P. sylvaticum 6	4	24.4 gh	−	−	+	+
P. sylvaticum 4	2	23.6 h	−	−	−	+

$^\alpha$ Values followed by the same letter/s are not significantly different from each other (P≤0.05) according to Bonferroni's test.

$^\beta$ + = isolate obtained; − = isolate not obtained

Pythium growth rates and colony characteristics

The diameters of colonies radiating from 1-cm-diameter plugs after 24 h on PDA for the different *Pythium* isolates ranged from 23.6 to 42.5 mm (Table 2). The fastest-growing isolates were *P. ultimum* RFLP type 1a, *P. ultimum* RFLP type 1b and *P. sylvaticum* RFLP type 2; the slowest-growing isolate was *P. sylvaticum* RFLP type 4. A possible relationship was observed between growth rates in axenic culture and the number of days after glyphosate treatment when these *Pythium* isolates were obtained from the roots of glyphosate-treated bean seedlings. There were no *Pythium* colonists in the root tissues prior to glyphosate treatment that were able to survive surface sterilization with NaOCl. With few exceptions, *Pythium* isolates with colony diameters of > 30 mm after 24 h in axenic culture (fast-growing) were those obtained at 2, 4, and 6 days after glyphosate treatment, whereas the *Pythium* isolates with < 29 mm colony diameters (slow-growing) were never obtained at 2 days but only at 4 and 6 days after glyphosate treatment (Table 2).

Pythium growth rates and RFLP grouping

Variance Components Estimation Procedure was conducted on the growth rates for all of the *Pythium* isolates assigned to each RFLP group to determine if all the isolates assigned within each RFLP group were indeed similar. The results (Table 3) indicated that 69% of the variation in growth rates was explained by species and RFLP groupings combined: 36% by species, and 33% by RFLP grouping. Another 22% of the variation was due to isolates assigned within the RFLP grouping and 9% to experimental error. Individual analyses for each species revealed that

Table 3. Results of Variance Components Procedure of colony diameters after 24 h in axenic culture for 65 *Pythium* isolates grouped within species, RFLP group within species, and isolates within RFLP group. The error term represents the variation of replicate plates within isolates.

Sources	SS	MS	Variance components	V:T [a]
Species	9394	2348.5	27.6	0.36
RFLP(species)	6063	603.6	25.1	0.33
Isolate (RFLP)	4152	83.0	16.7	0.22
Error	1963	7.2	07.2	0.09
Total	21545	3042.3	76.7	

[a] ratio of variance components and total variance.

most of the isolate within RFLP variation came from *P. ultimum*. The RFLP classification in this case did not explain the variation in growth. However, for *P. irregulare*, there was no residual variation among isolates once the RFLP variation had been taken into consideration and there was very little variation in *P. sylvaticum*.

GSI of 15 *Pythium* isolates on bean seedlings

The effect of different *Pythium* isolates on glyphosate LD_{50} values on beans is shown in Figure 5. There were significant differences between the three experiments and the isolates; therefore, separate analyses were done for each experiment. Twelve of the 15 isolates significantly (P=0.05) enhanced the herbicidal activity of glyphosate on 2-week-old bean seedlings, and 50% mortality was observed at doses 5 to 238-fold less than those required to cause 50% mortality in the absence of *Pythium*. Plants inoculated with *P. sylvaticum* RFLP type 1 and *P. coloratum* RFLP types 1 and 2, required similar amounts of glyphosate (70-120 µg per plant) to kill 50% of the plant population as compared with treatments not inoculated with *Pythium*. In only one case out of three repeated experiments was the LD_{50} dose associated with any of these three isolates were significantly less than the LD_{50} dose of the control plants. Plants inoculated with *P. sylvaticum* RFLP types 2,3,4,5 and 6, *P. irregulare* RFLP types 1 and 2, *Pythium* 'HS' RFLP types 1 and 2, *P. ultimum* RFLP types 1b and 2 all required less than 20 µg glyphosate per plant to kill 50% of the plant population. The plants inoculated with *P. ultimum* type 1a required the least amount of glyphosate to achieve 50% mortality (Figure 5).
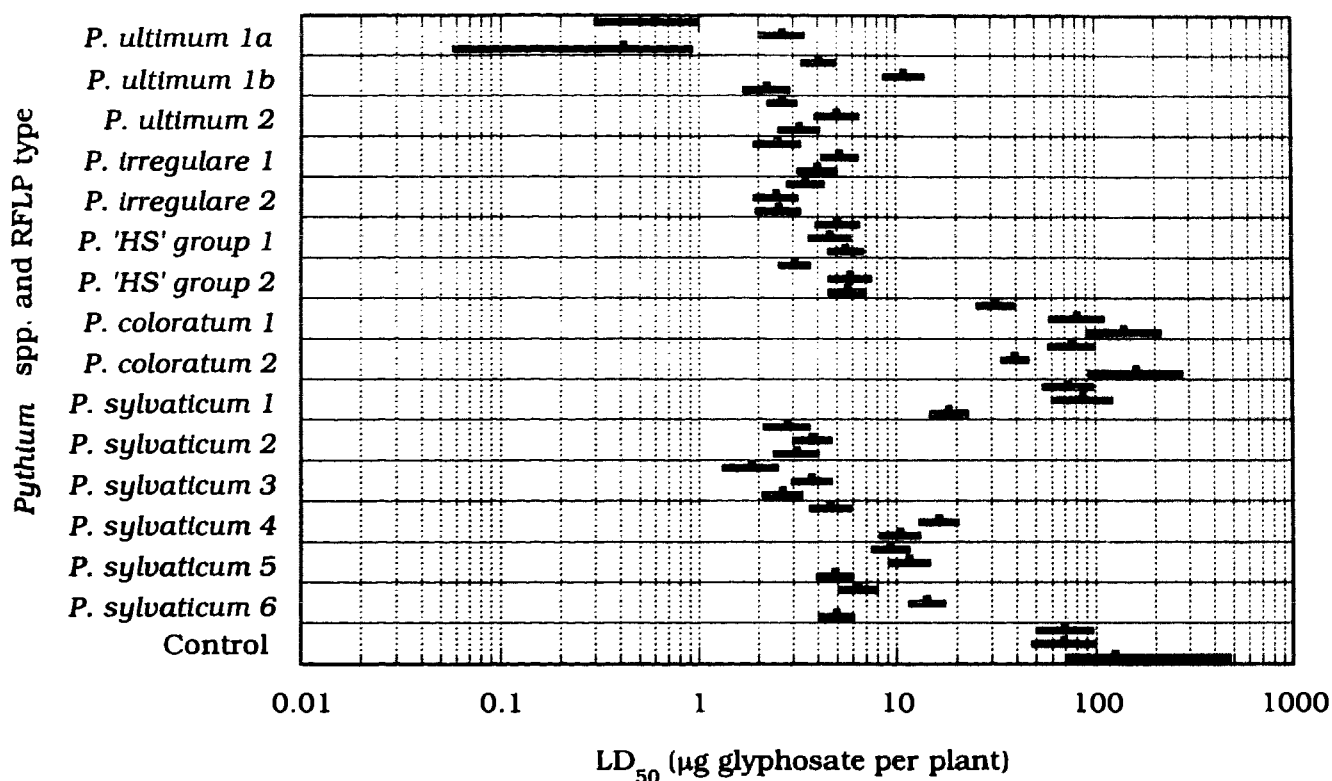
Figure 5. Effect of isolates of various species of *Pythium* obtained from
the roots of glyphosate-treated beans on glyphosate LD_{50} on
bean seedlings. Means and standard errors from three
experiments are shown on each line.

Pathogenicity Tests

The results from pathogenicity tests with the 15 different *Pythium*

isolates on beans showed significant differences in virulence (Table 4).

Symptoms included total seed rot, root rot and watery decay of seedling

cotyledons. Seedlings that managed to emerge had short roots and

noticeable stem girdling. All isolates of *P. ultimum* and *P. irregulare* were

highly virulent, causing 100% and 88-98% pre-emergence damping-off

on beans, respectively. *P. sylvaticum* RFLP types 2 and 3 and *Pythium*

'HS' group were moderately virulent, causing 35-52% pre-emergence

damping-off. Other *P. sylvaticum* RFLP types and all isolates of *P.*

coloratum were moderately to weakly virulent, causing a range of pre-

emergence damping-off from as high as 27% to as low as 9%. The 15

different *Pythium* isolates were less pathogenic on 2-week-old bean

plants than on germinating seeds. In both evaluations (seedlings in pots

and control treatments from GSI tests), *P. ultimum* RFLP type 1a caused

the highest post-emergence damping-off (28%). Isolates of *P. irregulare*

and other isolates of *P. sylvaticum*, *P. ultimum* and *Pythium* 'HS' group

caused post-emergence c amping-off ranging from zero to 15%. Some

isolates of *P. sylvaticum*, *Pythium* 'HS' group, and all isolates of *P.*

coloratum failed to cause post-emergence damping-off on beans. The

most noticeable symptoms in the post-emergence tests were brown stem

necrosis occurring at the soil line and progressing up the plant stem, and

wilting of the leaves.

Table 4. Mortalities observed in pathogenicity tests for different *Pythium* isolates on bean seedlings (cv Topcrop).

Pythium RFLP types	% Damping-off Mortality [a]		
	pre-emergence	post-emergence	
P. ultimum type 1a	100 a [β]	28 a [β]	29 a [δ]
P. ultimum type 1b	100 a	10 ab	13 cb
P. ultimum type 2	98 a	6 ab	9 cbd
P. irregulare type 1	88 a	7 ab	7 cbd
P. irregulare type 2	95 a	11 ab	15 b
P. 'HS' type 1	35 bcde	0 b	0 d
P. 'HS' type 2	37 bcd	1 b	6 cd
P. sylvaticum type 3	45 bc	9 ab	13 cb
P. sylvaticum type 2	52 b	4 ab	8 bcd
P. sylvaticum type 6	14 ef	6 ab	12 bc
P. sylvaticum type 4	10 f	2 ab	3 cd
P. sylvaticum type 1	15 def	0 b	0 d
P. sylvaticum type 5	10 f	3 b	6 d
P. coloratum type 1	27 cdef	0 b	0 d
P. coloratum type 2	9 f	0 b	0 d

[a] Values in columns, followed by the same letter/s are not significantly different from each other (P≤0.05) according to Bonferroni's test.

[β] Columns represent the means of three trials with 20 seeds or plants used for each isolate tested per trial; mortality recorded 1 week (pre-emergence) or 2 weeks (post-emergence) after inoculation.

[δ] Data obtained from the rows of glyphosate-untreated 2-week-old plants grown in *Pythium*-infested soil used as control treatment in three GSI experiments; mortality recorded 4 weeks after inoculation.

DISCUSSION

Morphological characteristics of *Pythium* isolates were inadequate for separating the different *Pythium* genotypes isolated from roots of glyphosate-treated bean seedlings growing in diverse soils. This was evident from the analyses of total DNA RFLP patterns, using either *Hind* III or *EcoR* I restriction enzymes, which showed that several morphologically similar *Pythium* isolates had different RFLP patterns, suggesting different genotypes. The use of RFLP patterns reduced the number of isolates needed to test for glyphosate synergistic interactions to a manageable number without omitting various genotypes represented within a particular *Pythium* species. Comparisons of RFLP patterns also were useful in determining the species affinities of isolates that did not produce sexual structures, particularly in *Pythium* 'G' group. This isolate was found to have an RFLP pattern identical with that of *P. ultimum* RFLP type 1. A similar result was obtained by Huang *et al.*, (1992) and Lévesque *et al.*, (1993b). Huang *et al.*, (1992) suggested that isolates of *Pythium* 'G' group are probably *P. ultimum* that have lost the ability to form sexual structures.

Four species, namely *P. sylvaticum*, *P. ultimum*, *P. irregulare* and *Pythium* 'HS' group were found to be glyphosate synergists on beans. One isolate of *P. sylvaticum* was found to be non-synergistic on beans, which suggests that other *Pythium* species and genotypes may be involved in the GSI on beans. On the other hand, there were isolates from within species that were better synergists than others, as was seen among *P. ultimum* and *P. sylvaticum* isolates. *P. ultimum* RFLP type 1a was a stronger glyphosate synergist than was *P. ultimum* RFLP type 1b or *P.*

ultimum RFLP type 2. This result could well have been overlooked if molecular techniques for differentiating the genotypes of morphologically similar isolates had not been used to select the representative *Pythium* isolates for the GSI tests.

It appears that the fast-growing isolates of *Pythium* colonized the roots of glyphosate-treated plants sooner than did the slow-growing isolates. It is possible that growth rate would be a component of an isolate's ability to function as a glyphosate synergist.

Although each of the four agricultural soils tested yielded substantial numbers of *Pythium* isolates compared to the virgin soil, the distribution of isolates within the five different soils did not suggest any pattern of location specificity, since all of the soil sites yielded some glyphosate synergistic *Pythium* species. The diversity of *Pythium* species in the agricultural soils was higher than in the virgin soil. *P. ultimum* was widely distributed in all of the agricultural soils. This was not surprising, since *P. ultimum* is known to have a broad host range and worldwide distribution (Hendrix and Campbell, 1973). The presence of many and diverse *Pythium* species in agricultural soils suggests that the occurrence of GSP may be related to factors that favor *Pythium* survival in these soils, such as suitable hosts and favorable conditions for reproduction, which are absent in areas not used for intensive crop production.

The limited numbers of glyphosate synergistic *Pythium* in the virgin soil may account for the high LD_{50} for glyphosate on plants in raw VS compared to that in raw ARS, CRS, LS and MS (Lévesque and Rahe, 1992). This finding supports the previous observation regarding the role of soilborne fungi in GSI and the role of *Pythium* species as glyphosate synergists.

All *Pythium* species isolated from glyphosate-treated bean plants were found to be pathogenic to varying degrees on glyphosate-untreated bean seeds and on 2-week-old seedlings. This raises the question as to whether the soil ecosystem could be modified via the glyphosate-*Pythium* synergistic interaction. It is not known whether a build-up of glyphosate synergistic *Pythium* species would occur after extensive use of glyphosate for weed management or for pre-harvest use as a crop desiccant. Previous studies have shown that soilborne *Fusarium* spp. increased when weeds, crops, or volunteer plants were treated with glyphosate (Lévesque *et al.*, 1987). If *Pythium* species are also increased by this mechanism, the potential for root diseases might also increase. In fact, increases in seedling diseases following applications of glyphosate have already been reported on barley (Lynch and Penn, 1982; Blowes, 1987; Smiley *et al.*, 1992), which suggests the possibility of a build-up of soilborne pathogens after herbicide treatment on weeds. Although the reported significant increase in the soil population of *Fusarium* spp. in areas where various weeds were treated with glyphosate did not cause significant detrimental effects on crops sown later in the same area (Lévesque *et al.*, 1987), this might not be true for *Pythium* species which are aggressive pathogens of germinating seeds. It is therefore important to determine if *Pythium* populations in soil are affected by glyphosate-treated plants, and to assess whether such an increase would result in greater incidence of damping-off on succeeding crops.

By using glyphosate-treated bean plants to isolate potential glyphosate synergistic *Pythium* from five diverse soils, it was found that of eight isolates of *P. irregulare*, seven were obtained from ARS, and that *P. irregulare* was the predominant species recovered from ARS. Braun

(1991) reported that *P. irregulare* is one of the causal pathogens of the apple replant disease in Nova Scotia. It is possible that *P. irregulare* may also be one of the pathogens involved in a similar disease in B.C. This coincidental observation suggests that glyphosate-treated plants might have potential as baits for selecting some of the fungal components involved in disease complexes such as apple replant disease where *Pythium* spp. are involved.

Our studies on GSI indicate that *Pythium* spp. are the predominant glyphosate synergists on bean seedlings. However, other soilborne fungi might also function as glyphosate synergists in nature. This possibility is supported by reports of increased occurrence of seedling diseases caused by fungi other than *Pythium* on crops planted in an area that was previously treated with glyphosate, such as *Rhizoctonia* spp. in barley (Smiley *et al.*, 1992), *Fusarium culmorum* in barley (Lynch and Penn, 1982), and *Gaeumannomyces graminis* in wheat (Mielke, 1983).

The research described here confirms the phenomenon of synergistic interaction of soilborne *Pythium* with glyphosate. It also shows that GSI involving different species of *Pythium* can be quantified by comparing the glyphosate LD_{50} values obtained from *Pythium*-inoculated and uninoculated plants. It is concluded that several different *Pythium* species can be glyphosate synergists on beans. However, it is also evident that some isolates within the same species or group are more efficient glyphosate synergists than others. The GSP were widely distributed in the diverse soils tested.

Since the different GSP identified were also pathogenic to glyphosate-untreated plants, and because it is probable that their populations will be affected when vegetation growing in an area is treated

with glyphosate, it is important to assess the long-term effects of glyphosate-treated plants on the dynamics of the *Pythium* populations in soil and to determine whether there is a pathogenic impact on crops subsequently planted in the same area.

Chapter III

ASSESSMENT OF HOST SPECIFICITY AMONG DIFFERENT SPECIES OF GLYPHOSATE SYNERGISTIC *PYTHIUM*

INTRODUCTION

Soilborne species of *Pythium* have been shown to augment the herbicidal activity of glyphosate by colonizing the roots of glyphosate-treated plants. This enhancement of herbicidal efficacy was termed glyphosate synergistic interaction (GSI) by Johal and Rahe (1984). Soilborne *Pythium* spp. were found to be the first and predominant root colonizers of glyphosate treated plants grown in different soils (Lévesque *et al.*, 1993a). Several species of *Pythium* are involved in GSI on bean (Descalzo *et al.*, 1996a) and on apple seedlings (Lévesque *et al.*, 1992). A possible indication of host-specificity of some glyphosate synergistic *Pythium* (GSP) was suggested from the result of an experiment by Lévesque *et al.* (1992), which showed that when *Pythium* isolates collected from the roots of glyphosate-treated apple and bean seedlings were tested for glyphosate synergistic interaction on apple seedlings, only the *Pythium* isolate from apple was glyphosate synergistic on apple seedlings. No other tests conducted so far substantiate this initial suggestion of host specificity among GSP.

This chapter describes the results from research to address the host specificity of GSP. The specific objectives were to determine if: a) isolates of *Pythium* species originating from the roots of 2-week-old glyphosate-treated bean and wheat seedlings were selectively synergistic on the host species from which they were isolated; b) isolates of *Pythium* species from the roots of glyphosate-treated bean seedlings were capable

of GSI on various unrelated dicot species; c) isolates of *Pythium* species from various glyphosate-untreated hosts were capable of GSI on bean seedlings; d) the relative virulence of isolates of various *Pythium* species differed on glyphosate-untreated hosts; and e) *Pythium* growth rates *in-vitro* and *Pythium* virulence in normal plants were correlated with their efficacy as glyphosate synergists.

MATERIALS AND METHODS

Sources of *Pythium* isolates

Isolates of five different *Pythium* species previously collected from the roots of glyphosate-treated bean and wheat seedlings grown separately in various soils were used. The *Pythium* isolates from glyphosate-treated beans were from the experiment described in Chapter 1, while the isolates from glyphosate-treated wheat were from the experiment by Lévesque *et al.*, (1993b). These isolates are referred to hereafter as *Pythium* isolates from bean (PIB) and *Pythium* isolates from wheat (PIW), respectively. The *Pythium* genotypes were defined on the basis of restriction fragment length polymorphisms (RFLP) of the total DNA (Descalzo *et al.*, 1996a; Lévesque *et al.*, 1993b). Letters B and W were added at the end of the isolates' names to indicate the original host (bean and wheat seedlings) from which the *Pythium* isolates were obtained. The number of genotypes represented per *Pythium* species tested are shown in Table 5.

Isolates of other *Pythium* species were obtained from the culture collections of Dr. Donald J.S. Barr (Biosytematics Research Institute, Ottawa, Canada) and Dr. Gloria Abad (North Carolina State University,

Table 5. *Pythium* species and the numbers of genotypes represented among isolates obtained from the roots of glyphosate-treated bean (PIB) and wheat (PIW) seedlings.

Pythium species	Number of genotypes obtained from	
	beans [α]	wheat [β]
P. ultimum	3	4
P. sylvaticum	6	7
P. coloratum	2	2
P. irregulare	2	1
P. 'HS' group	2	0
Total	15	14

[α] Bean seedlings were grown in various soils collected from the Okanagan and Fraser Valleys, B.C., Canada.

[β] Wheat seedlings were grown in various soils collected from the Fraser Valley, B.C., Canada.

Raleigh, N.C.). Except for *P. acanthicum*, which was isolated from soil, all other *Pythium* species in these collections were originally isolated from the roots of various host plants that were not treated with glyphosate (Table 6). This group of isolates is referred to as *Pythium* isolates from various hosts (PIVH).

Determination of glyphosate LD_{50} values for plants grown in soils infested with various *Pythium* isolates

To assess the relative efficacy of *Pythium* isolates to act as glyphosate synergists on plant species from which they were originally isolated, GSI tests were conducted using PIW on wheat seedlings (*Triticum aestivum* L. cv. Northstar Winter Wheat) and the results of GSI tests of PIB on bean seedlings (*Phaseolus vulgaris* L. cv. Topcrop) obtained in Chapter 2 were used to represent the effect of PIB on bean seedlings for this Chapter. Reciprocal GSI tests were then conducted using PIB on wheat and PIW on bean seedlings to determine whether the *Pythium* isolates were specific glyphosate synergists for the host plant species from which they were initially isolated. GSI tests were also done using PIB on two dicot hosts: sunflower (Family: Heliantheae, *Helianthus annus* L. cv. Sunwheat 101) and pepper (Family: Solanaceae, *Capcicum frutescens* L. cv. California Wonder). GSI tests of PIVH on bean seedlings were done to determine whether *Pythium* isolates obtained from various sources without the influence of glyphosate were capable of glyphosate synergistic interaction. To confirm whether the GSI test result obtained from wheat was a representative response of monocot plants, GSI test was done using PIB on corn seedlings (*Zea mays* L. cv. Early Golden Bantam).

Table 6. Different *Pythium* species obtained from various hosts and soil that were not treated with glyphosate (PIVH). α

Pythium species	Hosts/source	Geographic origin
P. aphanidermatum	English cucumber	B.C., Canada
P. sulcatum	Carrot	B.C., Canada
P. hypogynum	Lamb's-quarters	Quebec, Canada
P. coloratum	Alfalfa	Ontario,Canada
P. splendens	Blueberry	Nova Scotia, Canada
P. acanthicum	Soil	Ontario, Canada
P. arrhenomanes	Oat	Manitoba, Canada
P. spinosum	Turfgrass	North Carolina, USA
P. paroecandrum	Turfgrass	North Carolina, USA
P. vanterpooli	Turfgrass	North Carolina, USA

α *Pythium* isolates from Canada were provided by Dr. Donald J.S. Barr of Biosystematic Research Institute, Ottawa, Canada, and isolates from USA were from Dr. Gloria Abad, North Carolina State University, USA.

Except for the method of application and range of glyphosate doses used for monocot hosts, the methods used for all GSI tests were as described in Chapter 2. For monocot hosts, the glyphosate treatments were made as two, 1 μL droplets of aqueous dilutions of Roundup® (360 g a.i./L) applied in whorls of wheat and corn seedlings. A lower range of glyphosate doses (0, 0.5, 1.5, 5, 15, and 40 μg glyphosate a.i. per plant) were used for wheat and corn seedlings. To obtain 100% mortality at the highest glyphosate dose, *Pythium* uninoculated plants (controls) were treated with doses ranging from 0-60 μg glyphosate a.i. per plant. The GSI experiments done in this Chapter were repeated a minimum of two times.

Pythium growth rates *in-vitro* and pathogenicity tests

The ability of various *Pythium* isolates (PIB, PIW and PIVH) to cause pre-emergence damping off of germinating seeds and the isolate growth rates *in-vitro* were assessed using the procedures described in Chapter 2. Pathogenicity tests of the various isolates were done only for plant species that were used for GSI test involving the same isolate group. *Pythium* growth rates and virulence data for each isolate group were analyzed separately using ANOVA (SAS statistical package, 1985).

Correlation of *Pythium* growth rate, virulence and glyphosate LD$_{50}$

A simple linear correlation was performed separately for each of the *Pythium* groups' (PIB, PIW and PIVH) growth rate *in-vitro* vs their respective virulence and glyphosate LD$_{50}$ values obtained on various hosts tested. An arcsine and logarithmic transformation was done on the virulence and glyphosate LD$_{50}$ data, respectively, while untransformed

Pythium growth rate data were used in the correlation tests. Student's t-test was done to determine the significance of the Pearson product-moment correlation coefficient (Zar, 1984). The significant correlation results between growth rates *in-vitro* vs virulence were confirmed by doing an analysis of variance on the virulence data of *Pythium* isolates that were classified based on their colony diameter on PDA after 24h. The *Pythium* isolate groupings were: fast-(51-60 mm/24h), moderately fast-(26-50 mm/24h) and slow-(< 25 mm/24h) grower. A similar procedure was done to confirm the significant correlation obtained on *Pythium* growth rate vs the glyphosate LD_{50} values on bean.

The significant correlations obtained between virulence vs glyphosate LD_{50} values were also confirmed by analysis of variance. In this case, however, the various isolates were grouped based on their virulence on the various host tested. The following were the virulence groupings of the PIB on bean (highly virulent= 88-100%, moderately virulent= 35-87% and least virulent= 0-34% seedling mortality); PIB on pepper (highly virulent= 100%, moderately virulent= 72-99% and least virulent= 0-30% seedling mortality); and PIW on bean (highly virulent= 85-100%, moderately virulent= 24-84% and least virulent= 0-23% seedling mortality).

RESULTS

There were significant interactions between the replicated experiments and various *Pythium* isolates in all GSI tests. It was therefore necessary to analyse each experiment separately.

GSI effects of PIB on bean, wheat and corn seedlings

The effect of different *Pythium* isolates on glyphosate LD_{50} values on beans is presented and discussed in Figure 5, Chapter 2. The PIB GSI tests on wheat seedlings gave an inconsistent GSI response in three replicated experiments (Figure 6A). In two out of three experiments, plants inoculated with *P. irregulare* RFLP type B1; *P. sylvaticum* RFLP types B1 and 2; and *P. coloratum* RFLP type B2 had significantly (P=0.05) lower LD_{50} values than the control. In one out of three experiments, plants inoculated with *P. ultimum* RFLP types B1a and B1b; *P. irregulare* RFLP type B2; *Pythium 'HS'* group RFLP types B1 and B2; *P.sylvaticum* RFLP types B3, B4, B5 and B6; and *P. coloratum* RFLP type B1 had significantly lower LD_{50} values than the control. However, it was also observed that plants inoculated with *P. ultimum* RFLP type B2; *P. iregulare* RFLP type B2; *P. coloratum* RFLP B1; *Pythium 'HS'* group RFLP type B1; and *P. sylvaticum* RFLP types B3 and B6; as well as those inoculated with *P. ultimum* RFLP type B1b; *P. coloratum* RFLP type B2 and *P. sylvaticum* RFLP type B4 required similar amounts of glyphosate to kill 50% of the plant population as was needed for the control in one out of three and two out of three tests, respectively. It was also noted that in some instances plants inoculated with *P. ultimum* RFLP type B1a and B1b; *P. ultimum* RFLP type B2; *P. irregulare* RFLP type B1;
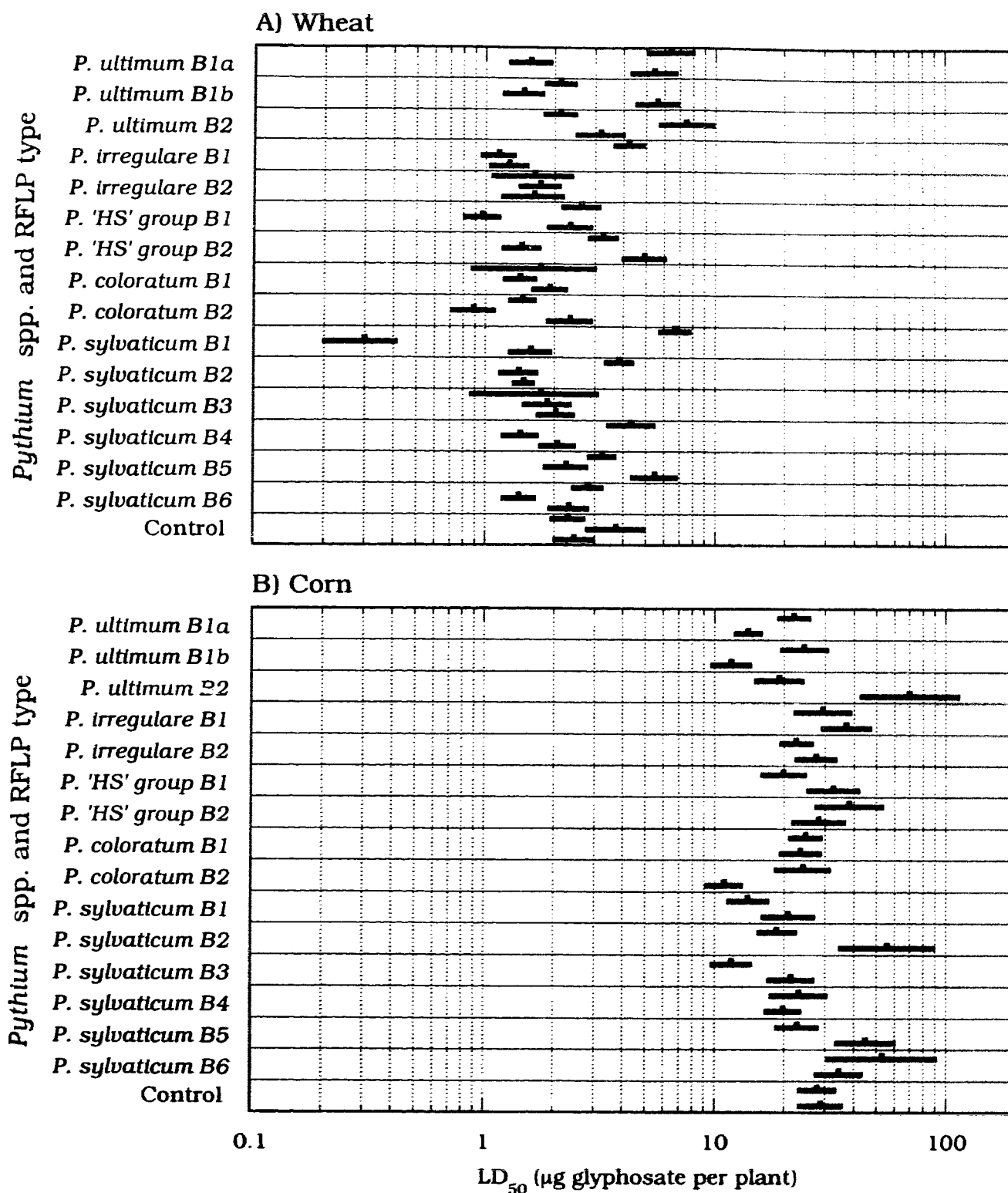
Figure 6. Effect of isolates of various species of *Pythium* obtained from the roots of glyphosate-treated beans on glyphosate LD_{50} on A) wheat and B) corn seedlings. Means and standard errors from three or two experiments are shown on each line.

P. sylvaticum RFLP types B1, B2, B4 and B5 had higher LD_{50} values than the control.

The GSI tests of PIB on corn seedlings also gave an inconsistent GSI response in two experiments conducted (Figure 6B). In one out of two experiments, plants inoculated with *P. ultimum* RFLP type B1a; *P. ultimum* RFLP type B1b; *P. coloratum* RFLP type B2; and *P. sylvaticum* RFLP types B1 and B3 had significantly (P=0.05) lower LD_{50} values than the control. Plants inoculated with *P. irregulare* RFLP types B1 and B2; *Pythium 'HS'* RFLP types B1 and B2; *P. coloratum* RFLP type B1; and *P.sylvaticum* RFLP types B4, B5 and B6 had similar glyphosate LD_{50} values with the control treatments in the two experiments conducted. In one of the two experiments done, it was also observed that plants inoculated with *P. ultimum* RFLP type B2 had higher LD_{50} values than the control.

GSI effects of PIW on wheat and bean seedlings

The effect of different PIW on glyphosate LD_{50} values on wheat seedlings is shown in Figure 7A. Plants inoculated with *P. ultimum* RFLP types W1, W2, W3, and W4; *P. coloratum* RFLP types W1 and W2; *P. sylvaticum* RFLP type W5 significantly (P=0.05) enhanced the herbicidal activity of glyphosate on 2-week old wheat seedlings in two replicate tests. In one out of two tests, plants inoculated with *P. irregulare* RFLP type W1; *P. sylvaticum* RFLP types W1, W2, W3, W4, W6 and W7 significantly enhanced the herbicidal efficacy of glyphosate on wheat seedlings. In one of two tests, plants inoculated with *P.sylvaticum* RFLP
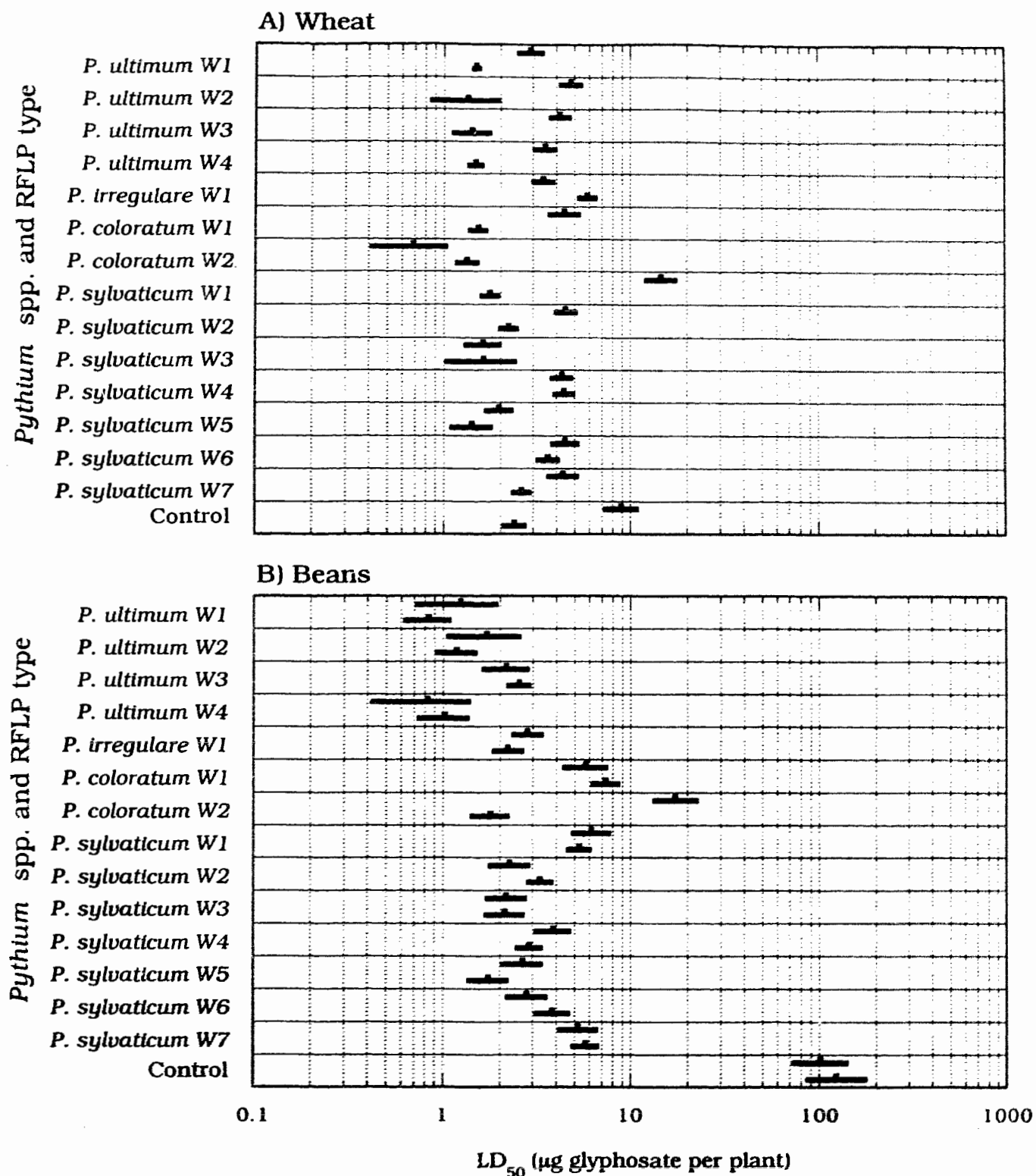
Figure 7. Effect of isolates of various species of *Pythium* obtained from the roots of glyphosate-treated wheat on glyphosate LD_{50} on A) wheat and B) bean seedlings. Means and standard errors from two experiments are shown on each line.

types W2, W3, and W7 needed similar amounts of glyphosate to kill 50% of the plant population as was required in the control treatment. In some instances, plants inoculated with *P. sylvaticum* RFLP types W1, W4, and W6; and *P. irregulare* RFLP type W1 had higher LD_{50} than the control treatment.

The effect of PIW on glyphosate LD_{50} values on beans is shown in Figure 7B. All PIW significantly enhanced the herbicidal efficacy of glyphosate on 2-week-old bean seedlings in two experiments. Fifty percent mortality was observed at doses 6 to 178-fold less of those required to cause the same level of mortality in the absence of *Pythium* (control).

GSI effects of PIB on other dicot plant species

All PIB enhanced the herbicidal activity of glyphosate on 2-week-old sunflower seedlings by causing 50% mortality at doses 9 to 32-fold less than those required by the control (Figure 8A). All PIB except *Pythium 'HS'* group RFLP type B1 and *P. coloratum* RFLP type B2 enhanced the herbicidal activity on pepper seedlings by causing 50% mortality at doses 11 to 54-fold less than those needed by the control (Figure 8B). *Pythium 'HS'* group RFLP type B1 and *P. coloratum* RFLP type B2 caused smaller but significant reductions of glyphosate LD_{50} on pepper seedlings.

GSI effects of PIVH on bean seedlings

The effect of different *Pythium* species obtained from soil and from glyphosate-untreated plants on glyphosate LD_{50} values on beans are shown in Figure 9. All *Pythium* species tested significantly enhanced
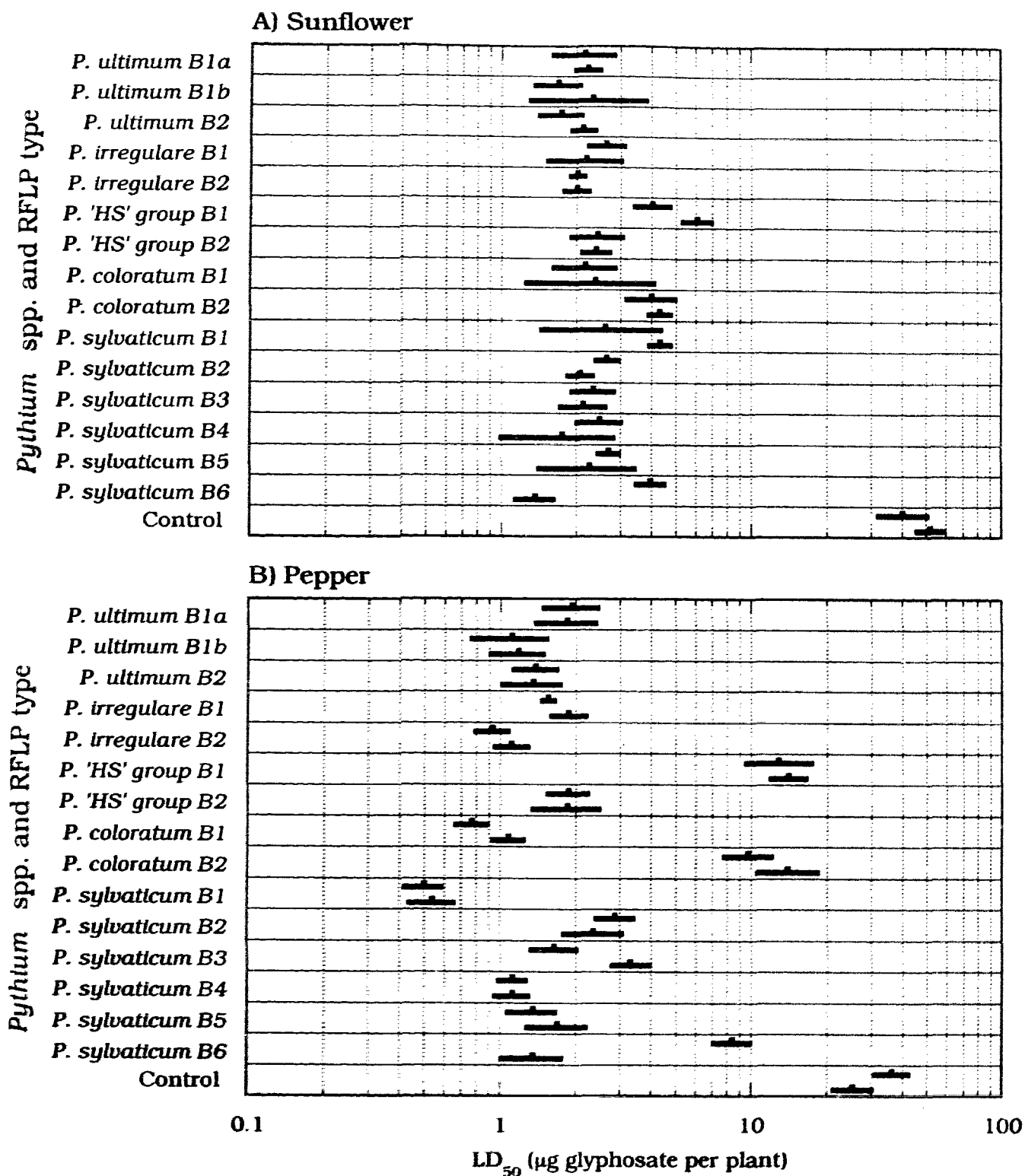
Figure 8. Effect of isolates of various species of *Pythium* obtained from the roots of glyphosate-treated beans on glyphosate LD_{50} on A) sunflower and B) pepper seedlings. Means and standard errors from two experiments are shown on each line.
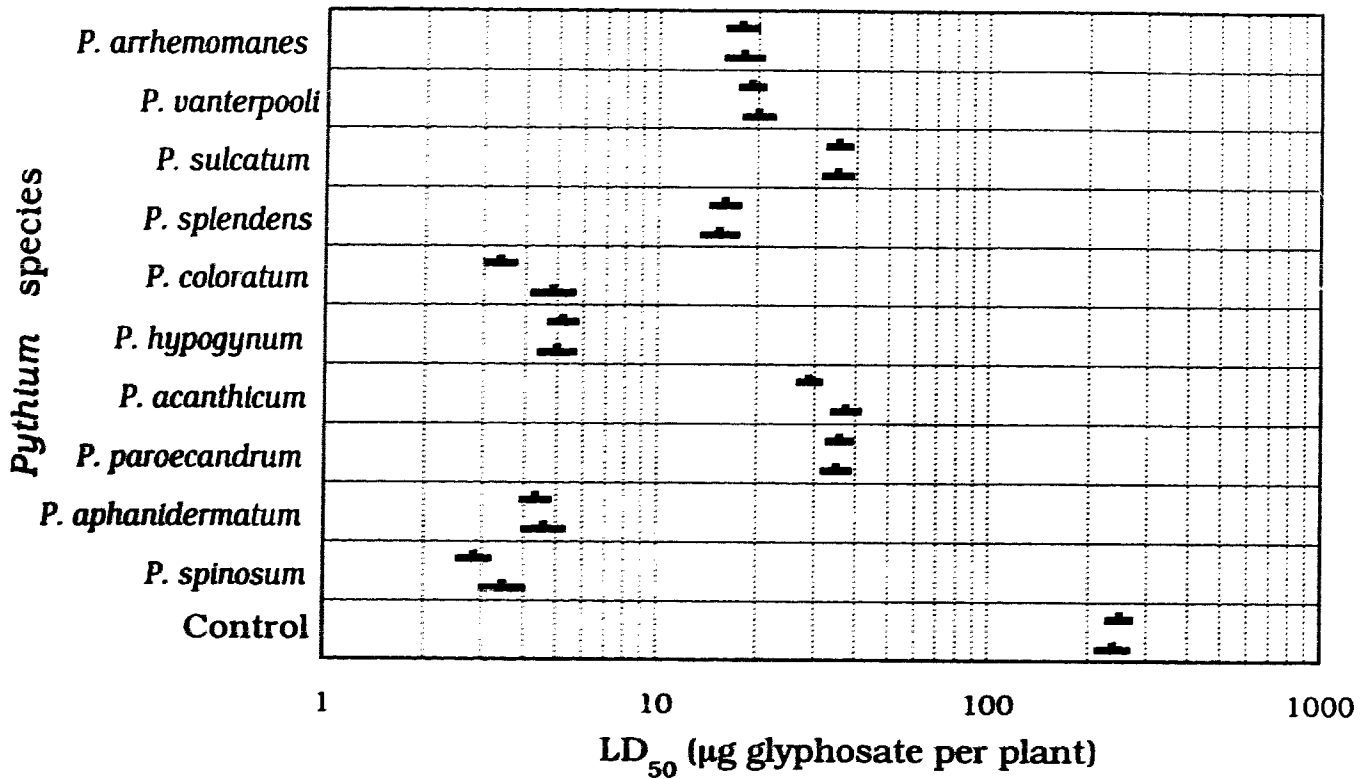
Figure 9. Effect of different species of *Pythium* obtained from various glyphosate-untreated hosts and soil on glyphosate LD $_{50}$ on bean seedlings. Means and standard errors from two experiments are shown on each line.

(P=0.05) the herbicidal effect of glyphosate on 2-week-old bean seedlings, and 50% mortality was observed at doses 7 to 80-fold less than those required to cause 50% mortality in the absence of *Pythium*. *Pythium coloratum*, *P. hypogynum*, *P. aphanidermatum*, and *P. spinosum* were significantly more effective as synergists than were other *Pythium* species in this group.

Virulence of PIB on germinating seeds of various plants

Symptoms of pre-emergence damping-off caused by various species of *Pythium* were similar in all types of plant tested. *Pythium* entered the germinating seeds in the soil by infecting the emerging radicle. After the initial infection, the mycelia colonized the growing embryo and cotyledons. Susceptible seeds were eventually killed and were covered with white mycelia 1 week after infection. Infected seeds seldom emerged from the soil, and those that were able to emerge from the soil were weak and had obvious stem necrosis immediately above the soil surface. Infected seedlings usually collapsed 3 to 4 days after emergence and showed symptoms of stem girdling.

The virulence of the PIB on germinating bean seedlings is shown and described in Table 4, Chapter 2. The same isolates were also virulent to varying degrees on germinating wheat seeds (Table 7). *P. ultimum* RFLP type B1a was the most virulent isolate, causing 95% pre-emergence damping-off. It was followed by *P. ultimum* RFLP types B1b and B2; *P. irregulare* RFLP types B1 and B2; *Pythium 'HS'* group RFLP type B2; and *P. sylvaticum* RFLP type B3, which caused pre-emergence damping-off ranging from 76% to 93%. *P. sylvaticum* RFLP types B1 and B2 were moderately virulent, causing 50 to 63% damping-off, respectively.

Table 7. Mortalities observed in pathogenicity tests on wheat, corn, pepper and sunflower seedlings using different *Pythium* isolates originally collected from the roots of glyphosate-treated bean seedlings (PIB).

Pythium species and RFLP type	% Pre-emergence damping-off mortality [α]			
	wheat	corn	pepper	sunflower
P. ultimum type B1a	95 a	96 a	100 a	35 ab
P. ultimum type B1b	91 ab	92 ab	100 a	23 abc
P. ultimum type B2	93 ab	92 ab	100 a	48 a
P. irregulare type B1	88 ab	98 a	100 a	25 abc
P. irregulare type B2	82 ab	40 abc	100 a	47 a
P. 'HS' group type B1	26 de	47 abc	15 c	3 c
P. 'HS' group type B2	84 ab	47 abc	100 a	2 c
P. sylvaticum type B1	50 cd	53 abc	100 a	22 abc
P. sylvaticum type B2	63 bc	60 abc	87 b	10 bc
P. sylvaticum type B3	76 abc	55 abc	72 b	27 abc
P. sylvaticum type B4	15 e	10 c	27 c	5 c
P. sylvaticum type B5	21 de	23 bc	90 ab	0 c
P. sylvaticum type B6	12 e	37 abc	30 c	5 c
P. coloratum type B1	10 e	57 abc	100 a	27 abc
P. coloratum type B2	9 e	9 e	100 a	10 bc

[α] Mean mortalities from three repeated trials, with three replicate plates used for each isolate per trial. Twenty seeds were used per plate and mortality recorded 2 weeks after inoculation. Values within column followed by the same letter/s are not significantly different from each other (P≤0.05) according to Bonferroni's test.

The least virulent isolates, *P. coloratum* RFLP types B1 and B2; *Pythium 'HS'* group RFLP type B1; and *P. sylvaticum* RFLP types B4, B5, and B6 caused pre-emergence damping-off ranging from 9 to 26%.

All PIB were also virulent to varying degrees on germinating corn seeds (Table 7). *P. irregulare* RFLP type B1 and *P. ultimum* RFLP type B1a were the most virulent isolates, causing 97 and 98% pre-emergence damping-off, respectively. *P. sylvaticum* RFLP types B4 and B5 were the least virulent isolates, causing pre-emergence damping-off ranging from 7 to 10%. The remaining isolates caused pre-emergence damping-off ranging from 23 to 92%, which was intermediate between the highest and the lowest % damping off obtained by PIB on germinating corn seeds.

The different PIB were virulent to varying degrees on germinating pepper seeds (Table 7). All isolates of *P. ultimum*, *P. irregulare*, and *P. coloratum* as well as *Pythium 'HS'* group RFLP type B2 and *P. sylvaticum* RFLP type B1 caused 100% pre-emergence damping-off. *P. sylvaticum* types B2, B3, and B5 caused mortalities ranging from 72 to 90%. *Pythium 'HS'* group RFLP type B1, and *P. sylvaticum* RFLP types B4 and B6 were the least virulent, causing 15 to 30% mortality on pepper seedlings.

PIB were generally less virulent to germinating sunflower seeds than to bean (Table 2), wheat, corn and pepper (Table 7). The most virulent isolates were *P. ultimum* RFLP type B2 and *P. irregulare* RFLP type B2, causing 48% and 47% mortalities, respectively. *P. ultimum* RFLP types B1a and B1b; *P. irregulare* RFLP type B1; *P. sylvaticum* RFLP type B1, B2, and B3; and *P. coloratum* types B1 and B2 caused mortalities ranging from 10 to 35%. Mortalities caused by the least virulent isolates,

Pythium 'HS' RFLP types B1 and B2, and *P. sylvaticum* RFLP types B4, B5, and B6, ranged from 0 to 5%.

Virulence of PIW on germinating wheat and bean seeds

All isolates of PIW were also virulent to varying degrees on germinating wheat seeds (Table 8). *P. ultimum* RFLP types W1, W2 and W3 were the most virulent, causing 95% mortality. *P. coloratum* RFLP type W1 and *P. irregulare* RFLP type W1 were the least virulent, causing 20% mortality. All the other PIW were intermediate in virulence, causing mortality ranging from 25 to 63%. When the same *Pythium* isolates were tested on beans, all except *P. coloratum* type W2 were virulent to varying degrees. *P. ultimum* RFLP types W1, W2, W3 and W4 were the most virulent, causing 85% pre-emergence damping-off while *P. coloratum* RFLP type W1, *P. irregulare* RFLP type W1, and *P. sylvaticum* types W2 and W6 were the least virulent, causing 8 to 23% mortality. *P. sylvaticum* types W1, W3, W4, W5 and W7 were intermediate in virulence, causing 25 to 45% mortality on germinating bean seeds.

Virulence of PIVH on germinating bean seeds

The varying virulence of PIVH on germinating bean seeds are shown in Table 9. *P. aphanidermatum* caused the highest pre-emergence damping-off (94%), followed by *P. spinosum* (66%) and *P. splendens* (27%), *P. paroecandrum* (19%) and *P. arrhenomanes* (5%). *P. coloratum*, *P. vanterpooli*, *P. hypogynum*, *P. sulcatum* and *P. acanthicum* isolates did not cause mortality on germinating bean seeds.

Table 8. Mortalities observed in pathogenicity tests on germinating wheat and bean seeds using *Pythium* isolates obtained from roots of glyphosate-treated wheat seedlings (PIW).

Pythium species and RFLP type	% Pre-emergence damping-off mortality [a]	
	wheat	beans
P. ultimum type W1	95 a	85 a
P. ultimum type W2	95 a	85 a
P. ultimum type W3	95 a	85 a
P. ultimum type W4	62 b	85 a
P. irregulare type W1	20 d	8 cd
P. sylvaticum type W1	48 bc	25 b
P. sylvaticum type W2	63 b	23 cd
P. sylvaticum type W3	60 b	45 ab
P. sylvaticum type W4	52 bc	35 b
P. sylvaticum type W5	25 cd	28 b
P. sylvaticum type W6	30 bcd	15 cd
P. sylvaticum type W7	45 bcd	42 ab
P. coloratum type W1	38 bcd	0 cd
P. coloratum type W2	20 d	15 cd

[a] Mean mortalities from three repeated trials, with three replicate plates used for each isolate per trial. Twenty seeds were used per plate and mortality recorded 2 weeks after inoculation. Values within column followed by the same letter/s are not significantly different from each other ($P \leq 0.05$) according to Bonferroni's test.

Table 9. Mortalities observed in pathogenicity tests on germinating bean seeds using isolates of *Pythium* species obtained from various glyphosate-untreated hosts and soil (PIVH).

Pythium species	% pre-emergence damping-off [α]
P. aphanidermatum	94 a
P. spinosum	66 b
P. splendens	27 c
P. paroecandrum	19 c
P. arrhenomanes	5 d
P. coloratum	0 d
P. vanterpooli	0 d
P. hypogynum	0 d
P. sulcatum	0 d
P. acanthicum	0 d

[α] Mean mortalities from three repeated trials, with three replicate plates used for each isolate per trial. Twenty seeds were used per plate and mortality recorded 2 weeks after inoculation. Values within column followed by the same letter are not significantly different from each other (P≤0.05) according to Bonferroni's test.

Pythium isolates growth *in-vitro*

Pythium isolates within PBI, PIW and PIVH groups showed varying growth 24 h after inoculation on PDA (Table 10). Among PBI group, the fastest-growing isolate was *P. sylvaticum* RFLP type B2 and the slowest-growing isolate was *P. sylvaticum* RFLP type B4. In PIW group, the fastest-growing isolate was *P. ultimum* type W1 and the slowest-growing isolate was *P. sylvaticum* RFLP type W3. In PIVH, *P. spinosum* and *P. sulcatum* were the fastest and the slowest-growing isolates, respectively. Other isolates within PBI, PIW and PIVH groups had colony diameters which were intermediate compared to the growth of the fastest- and slowest-growing isolates within their respective groups.

Correlation of growth, virulence and glyphosate LD_{50} values

There was a positive relationship between *Pythium* growth rates and virulence among all *Pythium* isolate groups on bean, wheat, corn, sunflower and pepper (Figure 10). However, significant correlations of these factors were only obtained with PIB on beans (r=0.69), wheat (r=0.75) and corn (r=0.68). These significant correlations were not confirmed by the results of an analysis of variance on the differences in virulence of the various *Pythium* isolates that were grouped under fast, moderately-fast and slow-growing categories.

Correlation analysis of *Pythium* growth rate and glyphosate LD_{50} values on various hosts tested generally showed a negative relationship (Figure 11). However, only the correlation result obtained from PIVH on beans was significant (r=-0.75). This significant correlation was not confirmed in the analysis of variance of the differences in the glyphosate LD_{50} values of isolates that were classified under fast-, moderately fast-

Table 10. Growth on PDA of various *Pythium* isolates collected from the roots of glyphosate-treated bean seedlings (PIB), glyphosate-treated wheat seedlings (PIW) and from various glyphosate-untreated hosts (PIVH) 24 h after inoculation.[a]

RFLP types of *Pythium* isolates from bean (PIB)	Growth (mm\24h)	RFLP types of *Pythium* isolates from wheat (PIW)	Growth (mm\24h)	Isolates of *Pythium* species from various hosts (PIVH)	Growth (mm\24h)
P. ultimum B1a	55.2 ab	*P. ultimum* W1	40.4 a	*P. aphanidermatum*	40.3 c
P. ultimum B1b	52.5 cb	*P. ultimum* W2	37.2 ab	*P. spinosum*	60.7 a
P. ultimum B2	49.7 cde	*P. ultimum* W3	33.8 ab	*P. splendens*	52.3 b
P. irregulare B1	44.3 f	*P. ultimum* W4	31.6 bc	*P. paroecandrum*	26.7 e
P. irregulare B2	46.1 ef	*P. irregulare* W1	26.1 cd	*P. coloratum*	41.9 c
P. 'HS' group B1	47.5 def	*P. sylvaticum* W1	36.3 a	*P. vaterpooi*	31.8 d
P. 'HS' group B2	51.2 cd	*P. sylvaticum* W2	38.6 a	*P. hypogynum*	41.6 c
P. sylvaticum B1	53.3 cf	*P. sylvaticum* W3	18.5 e	*P. sulcatum*	17.4 g
P. sylvaticum B2	59.0 a	*P. sylvaticum* W4	28.9 bc	*P. acanthicum*	28.2 e
P. sylvaticum B3	36.1 g	*P. sylvaticum* W5	34.7 ab	*P. arrhenomanes*	21.2 f
P. sylvaticum B4	24.3 i	*P. sylvaticum* W6	20.7 cde		
P. sylvaticum B5	33.8 gh	*P. sylvaticum* W7	29.1 bcd		
P. sylvaticum B6	17.6 j	*P. coloratum* W1	34.8 abc		
P. coloratum B1	35.5 g	*P. coloratum* W2	25.0 cde		
P. coloratum B2	31.5 h				

[a] Mean colony growth rate of *Pythium* isolates in six replicate petri plates. Values followed by the same letter/s are not significantly different from each other (P=0.05) according to Bonferroni's test.
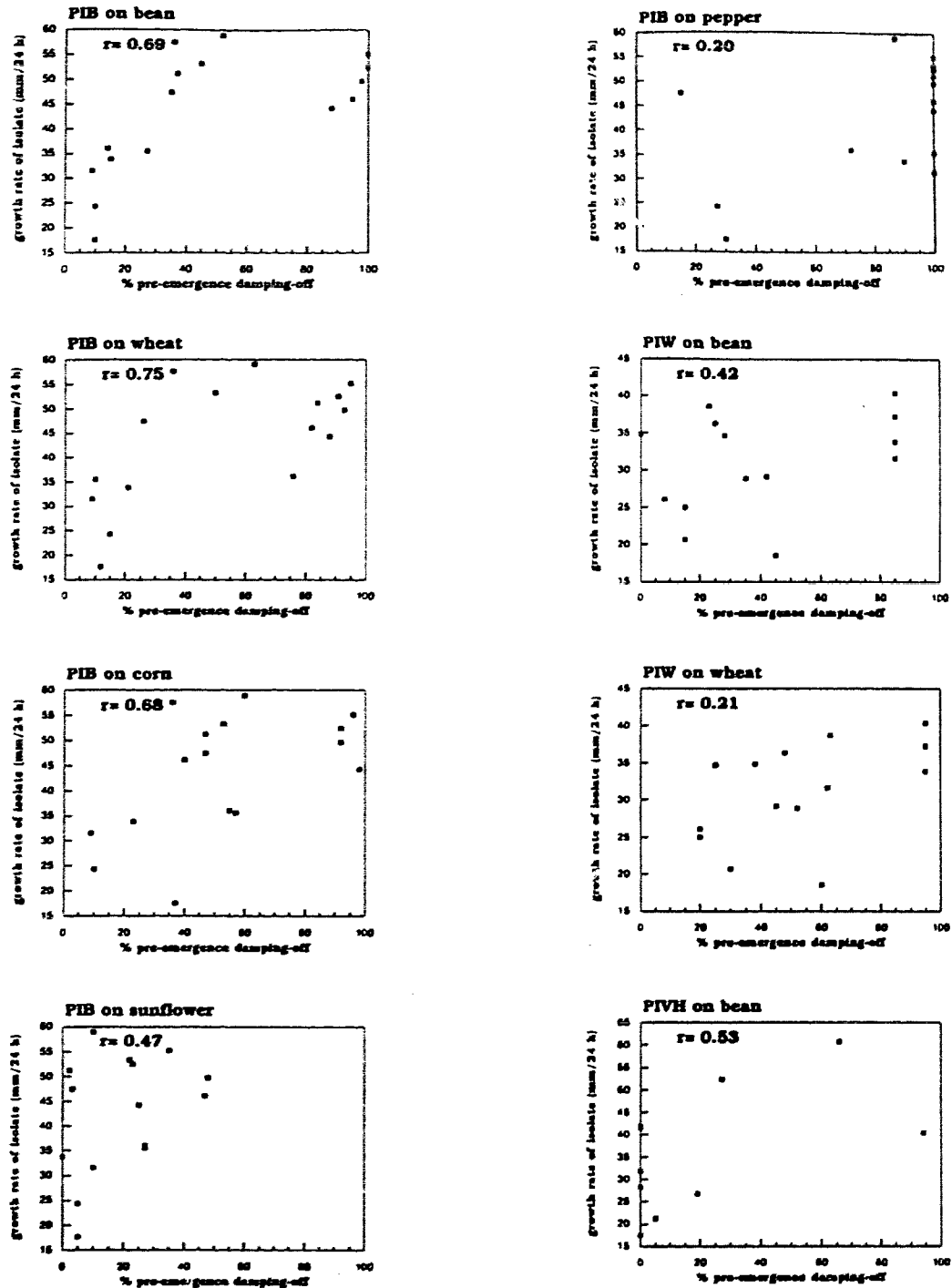
Figure 10. Correlation of growth rates (mm/24h) *in-vitro* of *Pythium* isolates from bean (PIB), *Pythium* isolates from wheat (PIW) and *Pythium* isolates from various hosts(PIVH) vs their percentage pre-emergence damping-off on different host species. Correlation coeffecients (r) with * are significant at P=0.05.
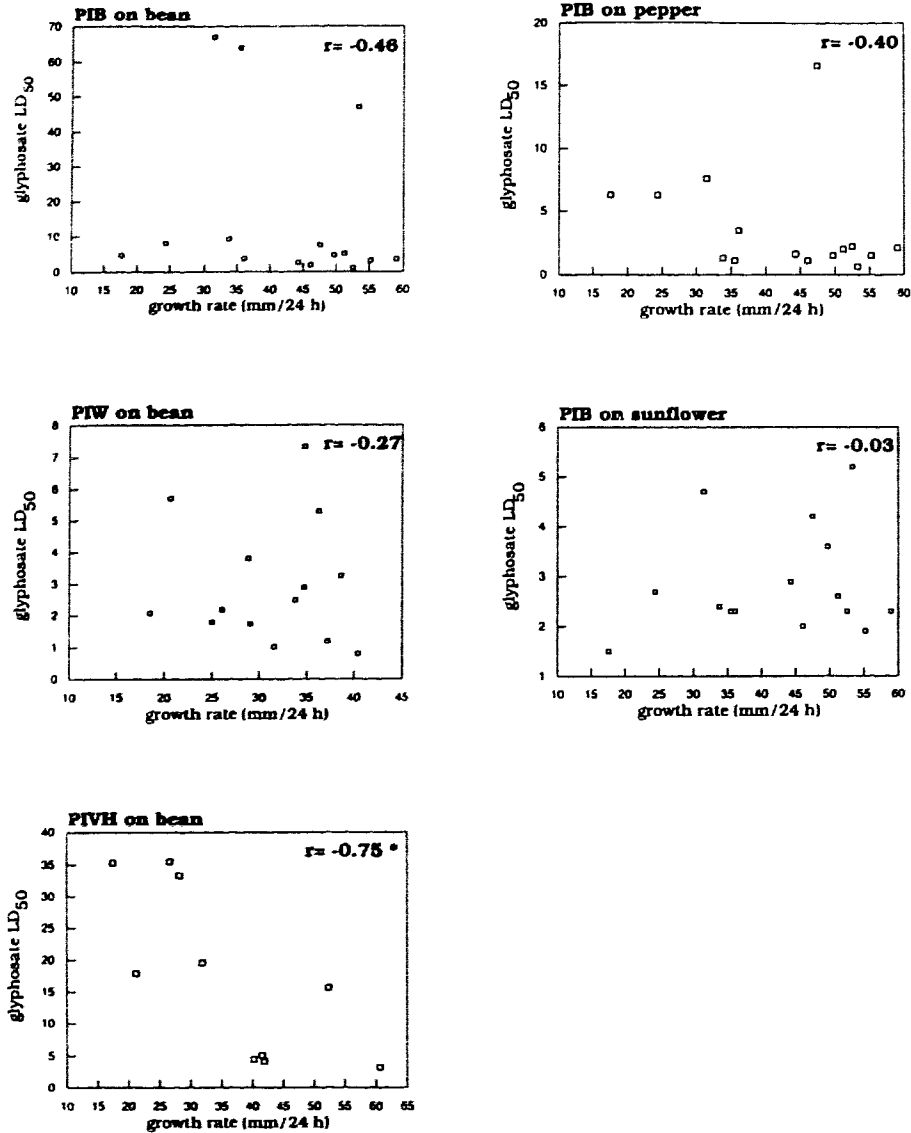
Figure 11. Correlation of the glyphosate LD_{50} values of *Pythium* isolates from bean (PIB), *Pythium* isolates from wheat (PIW) and *Pythium* isolates from various hosts (PIVH) on different dicot plant species tested vs the respective growth rates of these isolates on PDA. Correlation coefficient (r) with * is significant at P=0.05.

and slow-growing categories.

Correlation tests of virulence of *Pythium* isolates vs their glyphosate LD_{50} values on various hosts tested revealed a negative relationship (Figure 12), with significant results observed on PIB on bean (r=-0.69), PIW on bean (r=-0.68), and PIB on pepper (r=-0.76). Only the significant correlations observed in PWI on bean and PBI on pepper were confirmed by the significant results of the analysis of variance on the differences of the glyphosate LD_{50} values obtained by *Pythium* isolates that were grouped as highly-virulent, moderately virulent and least virulent. The glyphosate LD_{50} values obtained by the least virulent isolates were significantly differ ent (P=0.05) from those obtained by moderately and highly virulent isolates according to Tukey's test. The glyphosate LD_{50} values obtained by moderately virulent isolates and very virulent isolates were not significantly different from each other.

DISCUSSION

The variation in significant differences observed between repeated experiments illustrates the complexity of interactions between components of the soil ecosystem during GSI. Since it is impossible to control all variables in the soil ecosystem, conclusions regarding *Pythium*-plant species specificities during GSI must be based on the overall trend of several replicated GSI tests.

In general, the significance of GSI as evidenced by the magnitude of differences in glyphosate LD_{50} values obtained in the absence and presence of different *Pythium* isolates was greater on dicot than on monocot test plants.
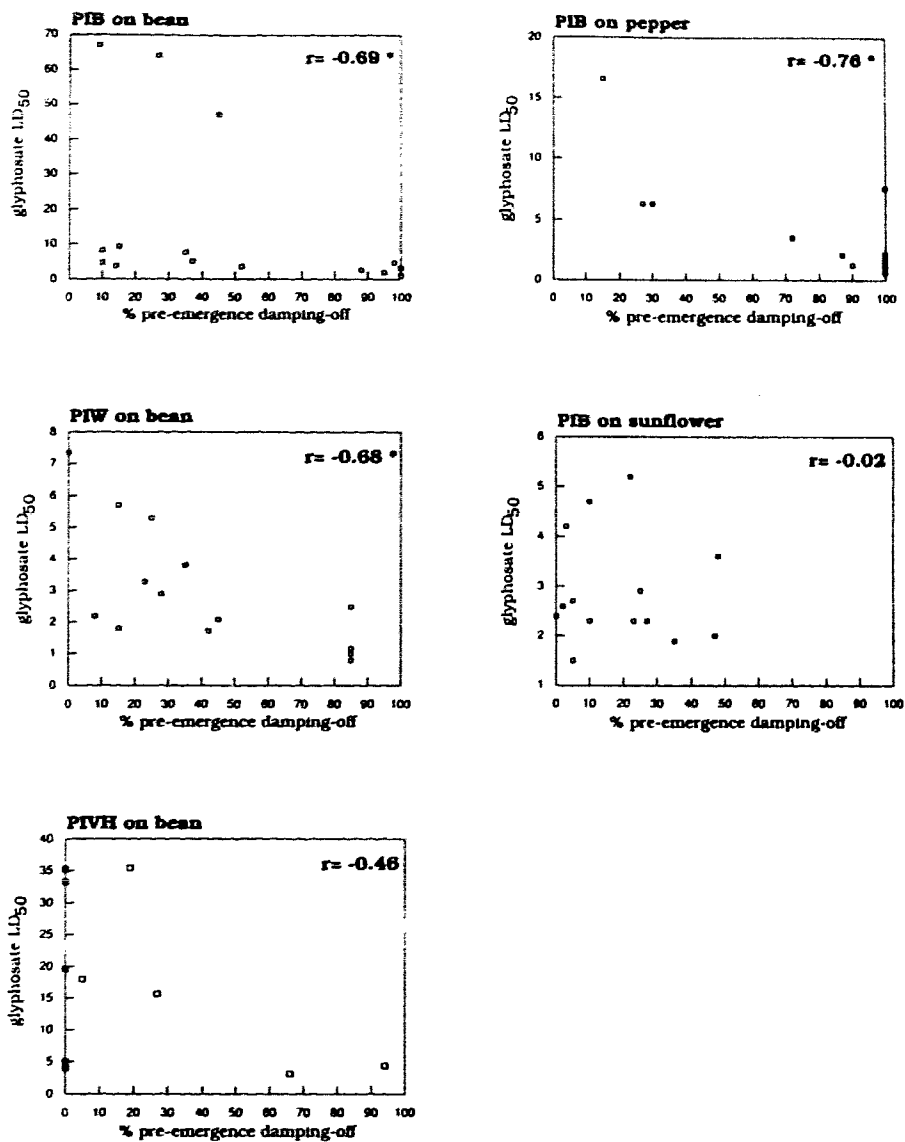
Figure 12. Correlation of the glyphosate LD_{50} values of *Pythium* isolates from bean (PIB), *Pythium* isolates from wheat (PIW) and *Pythium* isolates from various hosts (PIVH) on different dicot plant species tested vs their respective pre-emergence damping-off on the same host plants. Correlation coefficient (r) with * is significant at P=0.05.

This may be due to the substantial differences in glyphosate sensitivity observed between the two plant groups, as seen in the lower glyphosate dosages needed to cause equivalent plant mortalities on monocot than on dicot plant species.

GSI tests of PIW on wheat and reciprocal GSI tests of PIB on wheat and corn were not conclusive with regard to the determination of host specificity of these isolates as glyphosate synergists. However, a lack of specificity for the various herbaceous dicot hosts was clearly evident for *Pythium* isolates studied. All PIW were glyphosate synergists on bean seedlings, and all PIB were also glyphosate synergists on sunflower and pepper seedlings. Results from these tests showed no evidence for host specificity during GSI involving *Pythium* species on herbaceous dicot seedlings.

A differential GSI response was observed on apple seedlings for two isolates of *Pythium* (Lévesque *et al.*, 1992). In this experiment, the isolate obtained from beans was *P. ultimum*, but the isolate obtained from apple was not identified. It is possible that the contrasting GSI effect observed on apple seedlings was due to different *Pythium* species used. It is also likely that the differences between a woody and a herbaceous dicot species might result in specificities not observed among herbaceous dicot species. These possibilities notwithstanding, our results clearly support the conclusion that many different isolates and species of *Pythium* are capable of GSI with a general lack of specificity on diverse species of herbaceous dicot seedlings. This is not unexpected, given the wide host ranges of most *Pythium* species.

The *Pythium* species that were isolated from the roots of glyphosate-treated bean and wheat seedlings were generally similar

except for the presence of *Pythium 'HS'* group among PIB but not among PIW. Thus, the GSI tests that utilized *Pythium* species not represented in the PIB and PIW groups were needed to confirm whether other *Pythium* species were also capable of GSI. To date, 13 different *Pythium* species have been tested for GSI (five isolated from glyphosate-treated plants, eight from glyphosate-untreated hosts and soil). The finding that at least some isolates of all 13 species are capable of GSI suggests that other species of *Pythium* may also behave as glyphosate synergists on herbicide-treated plants in the field.

The different species of *Pythium* varied in their virulence to germinating seeds of herbaceous dicot species. This was an expected result since *Pythium* generally causes disease on diverse plant species, both monocot and dicot, ranging from herbaceous and woody (Hendrix and Campbell, 1973). *Pythium* causes different diseases depending on the growth stage of the host. Mature plants are affected by *Pythium* at root tips and root hairs, causing a subtle overall decline of plant health over time (Mircetich, 1971). Succulent and juvenile stem and root tissues of seedlings and germinating seeds are commonly attacked below the soil line, which results in girdling of the seedling stem and a watery decay of germinating seeds (Singh and Singh, 1984). The most prominent indication of severe *Pythium* attack on seedlings is manifested by damping-off, characterised by toppling-over of affected seedlings on the soil surface. *Pythium* can also be part of a disease complex on mature trees by causing synergistic interactions with other types of soil-borne microorganisms, as in apple replant disease (Braun, 1991), crown rot of apples trees (Jeffers *et al.,* 1982) and root rot of young apple trees (Utkhede and Smith, 1991).

The virulence of the various isolates of *Pythium* species from PIW and PIB groups on their respective reciprocal hosts showed indications of host preferences when the most virulent *Pythium* isolates were considered. The most virulent isolate of *P. ultimum* in the PIB group appeared to be more virulent on germinating bean seeds than on wheat. In contrast, the most virulent isolate of *P. ultimum* in the PIW group was less virulent on beans than on wheat. PIB were generally less virulent on sunflower than on beans or on pepper seeds. The presence of antifungal metabolites, (sesquiterpene lactones) in sunflower could have contributed to its resistance against *Pythium* (Spring *et al.*, 1982).

It was observed in Chapter 2 that fast-growing isolates of *Pythium* appeared to colonize the roots of glyphosate-treated plants sooner than did slower-growing isolates, suggesting a possible relationship between growth rate and virulence. This observation was not strongly supported by the correlation analyses of these factors in the three *Pythium* isolate groups tested. Therefore, *Pythium* growth rate is not a consistent predictor of *Pythium* virulence. *Pythium* growth rate was also inconsistent in predicting the isolates' glyphosate synergisic potential. It is probable that growth rate *in-vitro* may not reflect the rate of growth *in-vivo*.

Although significant correlations of virulence and glyphosate LD_{50} values of PBI on bean and pepper and PIW on bean seedlings were observed, inconsistent significant correlation results among the three *Pythium* isolate groups and hosts tested indicated that *Pythium* virulence could not predict an isolate's potential as a glyphosate synergist. This was clearly seen from the results of GSI tests conducted using PIVH. *P. coloratum* and *P. hypogynum* were non-pathogenic on germinating bean seedlings, but their efficacy as glyphosate synergists was similar to those

of *P. aphanidermatum*, the most virulent isolate. The ability of the non-pathogenic *Pythium* species to act as glyphosate synergist on bean seedlings clearly suggests that virulence is not the only factor involved in GSI. It is possible that the ability to produce pectinolytic enzymes may influence the efficacy of an isolate in colonizing the roots of glyphosate-treated plants.

The evidence that diverse *Pythium* species are capable of GSI and are generally non-host specific, at least on herbaceous dicot seedlings, and that these same isolates are pathogenic on germinating seeds highlights possible risks that might be associated with recurrent use of glyphosate as a herbicide and crop desiccant. Such risks might also be associated with the use of glyphosate on genetically engineered glyphosate-tolerant crops. There is both direct and indirect evidence that the population dynamics of soil microbes in the soil can be affected for short periods by glyphosate treatment (Lévesque and Rahe, 1987; Lévesque and Rahe, 1992; Smiley *et al.*, 1992). However, the meager published reports in relation to the extensive use of glyphosate-containing herbicides over the past two decades suggests that noticeable enhancement of root disease by glyphosate is not common. However, *Pythium* infections on mature plants are usually not discernable due to the insidious nature of pathogen development; as a result, any subtle effects on overall yield may go unnoticed. Research to assess the impact of glyphosate on long-term root rot disease potential of field soils and of sublethal doses on the activity of deleterious root microflora in perennial crops is clearly needed.

Chapter IV

GLPHOSATE TREATMENT OF BEAN SEEDLINGS CAUSES SHORT-TERM INCREASES IN *PYTHIUM* POPULATIONS AND DAMPING-OFF POTENTIAL IN SOILS

INTRODUCTION

Research conducted under laboratory conditions has identified soilborne fungi in the genera *Pythium* and *Fusarium* as the primary root colonists of glyphosate-treated bean and wheat seedlings (Lévesque *et al.*, 1993a). Results from pathogenicity tests with several *Pythium* isolates indicated that, ordinarily, root infection by *Pythium* spp. was tolerated by untreated 2-week-old bean, pepper, and sunflower seedlings. However, when comparable seedlings were treated with glyphosate doses that were sublethal in sterile soil, the combination of herbicide and *Pythium* resulted in plant death (Descalzo *et al.*, 1996b).

Poor establishment and growth of a succeeding crop has sometimes occurred in the field where glyphosate has been used to kill weeds before direct seeding of crops. Lynch and Penn (1982) attributed such effects or damage to toxins released from the decaying weed residues, as well as to increased inoculum potential of pathogens that were able to utilize the herbicide-treated weeds as substrates. Similar secondary herbicidal effects have been described in the Pacific Northwest of the USA and in Australia, where glyphosate-based herbicides are routinely used prior to direct seeding of cereals. Smiley *et al.* (1992) observed that glyphosate, when applied to kill volunteer cereals and weeds shortly before planting of spring barley, favored Rhizoctonia root rot of the newly planted crop. They hypothesized that the *Rhizoctonia* inoculum potential peaked within a few days after glyphosate treatment

and then declined. Roget *et al.* (1987) reported a similar pattern of disease in Australia asssociated with the application of paraquat + diquat + cyanazine. Blowes (1987) examined the causes of poor barley seedling emergence in Australia where root residues from glyphosate-treated ryegrass were present in the soil. While he concluded that plant root residues may have supported an increase of *Pythium* inoculum in moist soils, no measurements of *Pythium* populations were made before or after the ryegrass was treated. Instead, the increased infection of the barley seedlings by *Pythium* was used as evidence to support the conclusion that *Pythium* populations in the soil had been enhanced by the use of glyphosate. Lévesque *et al.* (1987) reported an increased number of colony forming units (CFU) of *Fusarium* spp. per g of soil in plots where glyphosate was used to kill weeds. However, they found that crops subsequently sown in these plots were not adversely affected.

Recently, Descalzo *et al.* (1996a) described the relative synergistic potential of various *Pythium* species on beans. Isolates of *P. ultimum* were among the most effective glyphosate synergists while *P. coloratum* isolates were non-glyphosate synergistic. Currently, there is no information on the effect of glyphosate-treated plants on the population dynamics of glyphosate synergistic *Pythium* (GSP) and non-GSP in soil. Such information is needed to assess the potential side effects of the use of glyphosate for weed control on crop lands, since several *Pythium* species are pathogens of germinating seeds, seedlings and mature plants (Van Der Plaats-Niterink, 1981).

This chapter describes the results of experiments to determine the effects of herbicide-treated plants on the population dynamics of *Pythium* in soil. The effect of root residues from glyphosate-treated bean plants on

the dynamics of the overall population of *Pythium* in field soil was investigated. In addition, the potential of glyphosate or paraquat to selectively increase the population of GSP in soil was compared by using root residues from plants treated with either herbicides and quantifying the populations of GSP and non-GSP isolates in autoclaved sandy loam soil. Damping-off pathogenicity tests were also done to determine whether changes in the populations of introduced *Pythium* would affect their pathogenic potential on germinating crop seeds.

MATERIALS AND METHODS

Effect of bean root residues on the general *Pythium* population in soil

About 100 kg of a muck (humic) soil (OM= 40%, pH= 4.0) were taken from a field near Cloverdale, B.C. that had previously been cropped to vegetables, and placed in a 100 L plastic container. The soil was kept moist and stored in the container at room temperature for about a month before it was passed through a 4.5 mm sieve to remove large organic debris. The soil was mixed thoroughly and 2.5 kg portions were placed in 2 L plastic pots. The following treatments were randomly assigned to individual pots: A) untreated soil, B) soil planted to beans, C) soil planted to beans and treated with glyphosate, and D) soil amended with heat-killed bean roots. Four replicate pots were used per treatment. The experiment was conducted once.

Bean seeds (*Phaseolus vulgaris* L. cv. Topcrop) were surface disinfected with 1% NaOCl solution for 5 min and washed three times with distilled water. Two seeds were sown in each of the pots assigned to

treatments B and C. Each pot was placed on a separate plastic dish to prevent cross contamination of leachate between the pots. Individual pots representing each treatment were randomly placed on 35x45 cm plastic trays (randomized complete block design) and kept in a growth room with a 16-h photoperiod and 25:19°C (day:night) temperature regime. The light intensity at plant height was 230 μmol m^{-2} s^{-1}. The soil was kept moist by daily addition of 100 mL of distilled water per pot. In lieu of distilled water, each pot received 100 mL of solution containing 0.004 g of 20-20-20 fertilizer per L of distilled water 1 week after seeding.

Two weeks after seeding, leaves of plants growing in pots of treatment C were sprayed with glyphosate (as Roundup® at 6 mL/L) until wet. Material was prepared for treatment D by collecting and killing the roots of 2-week-old bean plants grown in autoclaved Metro-mix™ by plunging them in boiling water for 10 sec and then air drying the roots overnight. One g of air dried roots was buried in the soil of each pot of treatment D in a 5-cm-deep hole in the center of the pot. The hole was then covered with soil from the same pot. Treatments D and C were applied at the same time.

Soil populations of *Pythium* were estimated at 0, 3, 6, and 10 days after herbicide treatment (DAHT) by dilution plating of soil samples from the pots on a *Pythium*-selective medium as described by Ali-Stayeh *et al.* (1986), but with vancomycin omitted. At each sampling time, approximately 25 g of soil was sampled from a depth of 15 cm at each of four equidistant locations in each pot using a soil core borer sterilized with 75% EtOH. The four samples per pot were mixed thoroughly and then two 5 g (fresh weight) sub samples were taken. One sub sample was dried at 90°C overnight and used to estimate the percentage of soil

moisture. The other sub sample was suspended in a 500 mL flask with 0.08 % water agar (WA) to bring the volume of the suspension to 250 mL, then the flask was placed on a rotary shaker for 30 min. Five mL of the resulting soil suspension were mixed with 95 mL of 0.08% WA to yield a 1:1000 dilution, and 1 mL aliqouts of this dilution were poured onto solidified *Pythium*-selective medium (Ali-Shtayeh *et al.*, 1986). The soil solution was spread evenly over the medium by rotating the plates. Six to eight plates were used per pot per sampling time. After incubation of the plates in the dark at 22-23°C for 24 h, the soil solution was washed off the surface of the plates under slowly running tap water and *Pythium* colonies were marked. The plates were then incubated for a further 24 h to allow slow-growing colonies to be located. Quantitative estimates of the number of propagules per g dry weight of soil were calculated by multiplying the mean number of *Pythium* colonies per plate by the dilution factor, and then the product was divided by the dry weight of the oven dried soil sample.

Effect of herbicide-treated plants on the population dynamics of GSP and non-GSP

Isolates of *P. ultimum* RFLP type 1a and *P. coloratum* RFLP type 1 used in this experiment were selected from a collection of *Pythium* isolates that were originally obtained from glyphosate-treated bean plants grown in different soils taken from various sites in British Columbia. The two selected isolates were a strong glyphosate synergist and a non-glyphosate synergist on bean plants, respectively (Chapter 2).

One hundred kg of sandy loam soil (OM= 12%, pH= 5.0) was taken from a field near Aldergrove, B.C., that had previously been cropped to

vegetables. The soil was kept moist and was passed through a 4.5 mm sieve to remove large debris. Batches of 2 kg of soil were put in autoclavable plastic bags and sterilized at 121°C for 45 min on each of 2 consecutive days. The soil was left in the sealed bags at room temperature (20-23°C) for 4 weeks before inoculation with either *P. ultimum* or *P. coloratum*. Inoculum was made by growing both fungi separately in stationary culture in V8-cholesterol broth (Ayers and Lumsden 1975) for 8 days. The mycelia were collected and rinsed with sterile distilled water on a Büchner funnel lined with filter paper. The mycelial mats were aseptically cut into nominal 1 cm^2 pieces with a scalpel, and suspended in 0.08 % sterile WA at 1 g fresh weight per 100 mL, and macerated in a Sorvall blender for 20 sec. Inoculation was done by mixing 10 mL of the resulting mycelial suspension per 150 g of soil (dry weight basis). The soil samples inoculated with the different *Pythium* species were aseptically transferred into separate 100 L disinfected plastic containers, covered with tight lids and kept at room temperature.

The progress of colonization of these soils by *P. ultimum* or *P. coloratum* was monitored using a soil plating procedure similar to that used above except that soil plating was done at 3-day intervals from day 0, until 42 days after inoculation (DAI). Aseptic technique was used when taking the soil samples from the 100 L containers at each sampling time, and when removing the soil needed for the treatment pots at 30 DAI.

Each of the *Pythium*-inoculated soils was mixed thoroughly in the separate containers after 30 days of incubation, then 2.5 kg portions were weighed into pots similar to those used in experiment 1. The following treatments were randomly assigned to individual pots: A) inoculated soil; B) inoculated soil planted with beans; C) inoculated soil

planted with beans and treated with glyphosate; and D) inoculated soil planted with beans and treated with paraquat. Each of the four treatments were used for *P. ultimum-* and *P. coloratum*-inoculated soil. Three replicate pots were used for each treatment.

Seeding and growing conditions used in this experiment were similar to those of the first experiment. Two weeks after the beans were seeded, the plants growing in pots C and D were treated with glyphosate (as Roundup® at 6 mL/L) and paraquat (as Gramoxone® at 9 mL/L) respectively, by spraying the leaves with the herbicide solution until they were wet. The soil populations of *Pythium* in each pot were determined at 0, 3, 6, 9, 12, 15, 18 and 21 days after herbicide treatment (DAHT) using the soil plating procedure described previously.

Pre-emergence damping-off pathogenicity tests were conducted at each soil sampling time, using soils from the same samples used to estimate *Pythium* populations. Ten g portions of soil were put into 1x5.5 cm Petri plates. Twenty seeds of surface-disinfected pepper (*Capsicum frutescens* L. cv. California Wonder) or sunflower (*Helianthus anuus* L. cv. Sunwheat 101), were sown on top of the soil. Three replicate plates were used per host. The seeded plates were watered using a hand-held sprayer with approximately 3 mL of distilled water per plate. The lids of plates were replaced and the seeded plates were placed in a tray lined with a single layer of moistened paper towel. The tray was covered completely with Saran wrap™ plastic film to minimize water evaporation during incubation for 1 week at 20-23°C. The numbers of dead and living seedlings were counted 1 week after seeding and the percent damping-off was computed.

Statistical Analysis

P. *ultimum* and P. *coloratum* populations were separately analyzed during each plating period since no simple linear or nonlinear models that would adequately describe the data could be found. Logarithmic transformation of population data were done before conducting the analysis of variance (ANOVA) in SAS statistical package (1985). Tukey's test was used to compare the differences between treatment means. ANOVA was also used on arcsine-transformed pre-emergence damping-off data for each plating period. Multiple comparisons between treatments were made using Tukey's test.

To determine the relationships between *Pythium* population levels and their pathogenic potential, *Pythium* population estimates were correlated with the correponding percent pre-emergence damping-off observed for each soil sample. Arcsine transformed damping-off data were used in the correlation tests.

RESULTS

Effect of various bean root residues on the general *Pythium* population in muck soil

The populations of *Pythium* in the pots comprising the various treatments at the start of the experiment ranged from 690 to 904 CFU per g of dry soil (Figure 13) which were not significantly different from one another. There were also no significant differences in the general *Pythium* population among the various treatments compared with the control at 3 and 10 DAHT. However, significant differences were observed at 6 DAHT, where the general *Pythium* populations in pots containing the
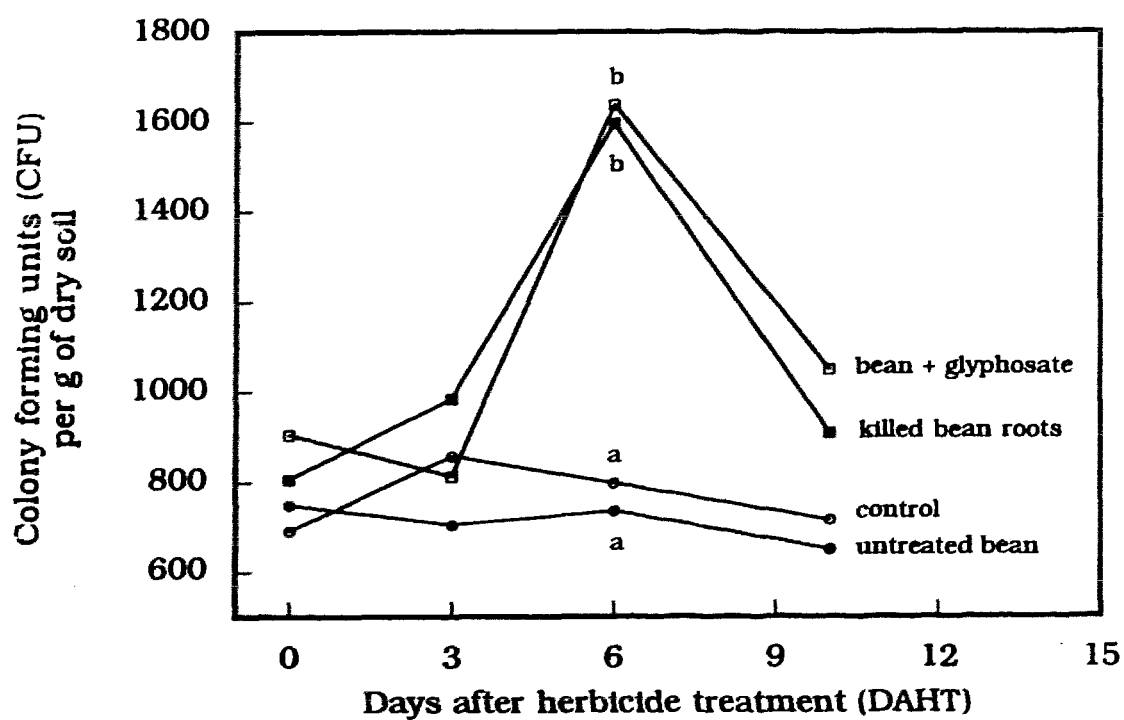
Figure 13. The effect of bean root residues on the general population of *Pythium* in potted muck soil. Symbols at 6 DAHT followed by the same letter are not significantly different from each other (P=0.05).

glyphosate-treated beans (C) and heat-killed bean roots (D) were significantly higher than with untreated beans (B) and control (A). The *Pythium* population in pots with untreated beans (B) was not significantly different from control (A).

Colonization of autoclaved soil by *P. ultimum* and *P. coloratum*

The estimates of CFU of *P. ultimum* and *P. coloratum* following introduction of these fungi into autoclaved sandy loam soil are shown in Figure 14. *P. ultimum* increased from 8 CFU per g of dry soil immediately after inoculation to 17 CFU and 165 CFU by 3 and 6 DAI, respectively. This was followed by fluctuations between 131 and 236 CFU during 9 to 42 DAI. The *P. coloratum* population in the soil increased from 95 CFU immediately after inoculation to 6861 CFU by day 9. The population level ranged between 6500 and 9500 CFU during the period 12 to 21 DAI and then declined to 1900 CFU at 27 DAI, and ranged between 1100 and 2100 CFU for the duration of the sampling period.

Symptom development on bean plants treated with herbicides

Untreated bean plants grown in both the naturally *Pythium*-infested muck soil and in the *P. ultimum*- or *P. coloratum*-inoculated sandy loam soil appeared healthy throughout the experiments. The glyphosate-treated bean plants in both experiments developed necrotic leaf spots within 2 days after treatment. Roots and stem tissues below the soil surface had rotted by 3 days after treatment in plants growing in the *P. ultimum*-inoculated soil, and the rot progressed rapidly upward and killed the plants by 4 DAHT. Root rot symptoms on glyphosate-treated plants growing in the *P. coloratum*-inoculated soil occurred 3 days
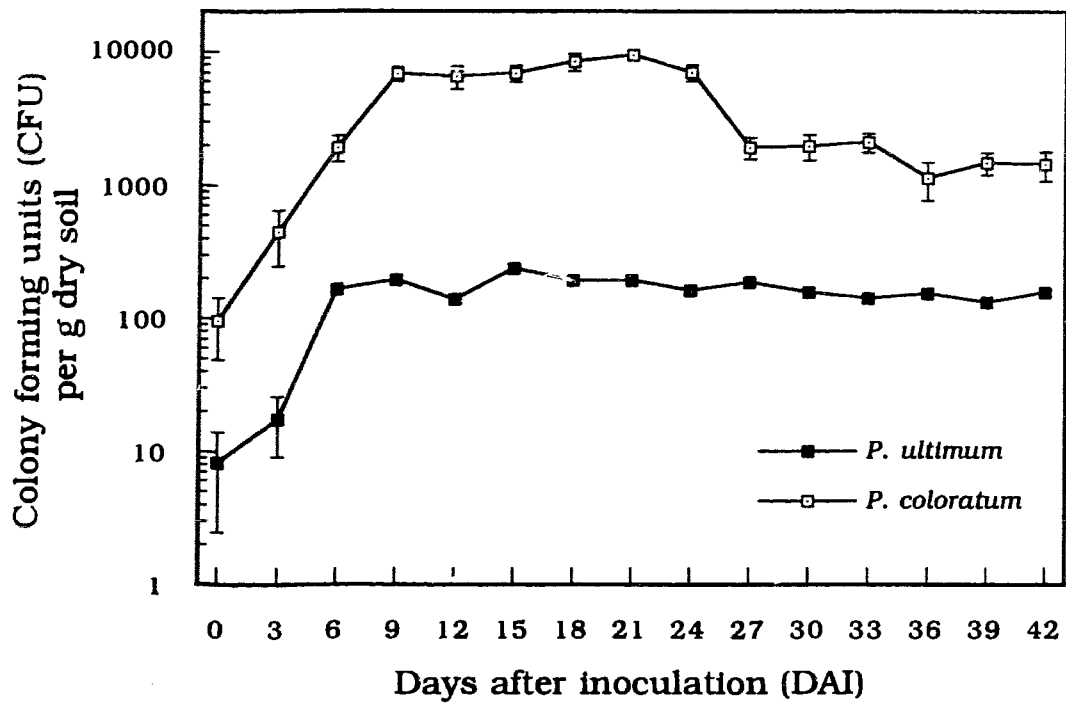
Figure 14. Population dynamics of *Pythium ultimum* and
P. coloratum inoculated into autoclaved sandy
loam soil.

later than those growing in *P. ultimum*-inoculated soil, and the plants died several days later.

In contrast, paraquat-treated bean plants in both the *P. ultimum*- and *P. coloratum*-inoculated soils showed drying of the leaves and stems 1 DAHT. The symptoms progressed to bleaching of the dry leaves and stems by 2 DAHT. The stems immediately above and below the soil surface appeared turgid and intact even at 6 and 9 DAHT, but root rot and collapse of the stems below the soil surface occurred by 12 to 15 DAHT. The dry wilted leaves of paraquat-sprayed plants were noticeably bleached in appearance, in contrast to the wilted leaves of the glyphosate-sprayed plants which remained green.

Effects of herbicide-treated plants on population dynamics of *P. ultimum* and *P. coloratum*

Soil populations of *P. ultimum* were generally enhanced approximately 10-fold over the control in the treatments involving bean roots from untreated bean plants, glyphosate-treated bean plants and paraquat-treated bean plants during the period 6-9 DAHT. A similar but slower and less uniform response was seen for *P. coloratum* (Figure 15). No consistent differences in the responses to the three treatments involving bean roots were evident, although a distinct population peak in the bean + glyphosate treatment was evident at 9 DAHT for *P. ultimum*, and at 9, 12 and 15 DAHT for *P. coloratum*.

Virulence of *P. ultimum* and *P. coloratum* on germinating pepper and sunflower seeds

Pre-emergence damping-off of germinating pepper seeds that were planted in *P. ultimum*- or *P. coloratum*-inoculated soil of the various
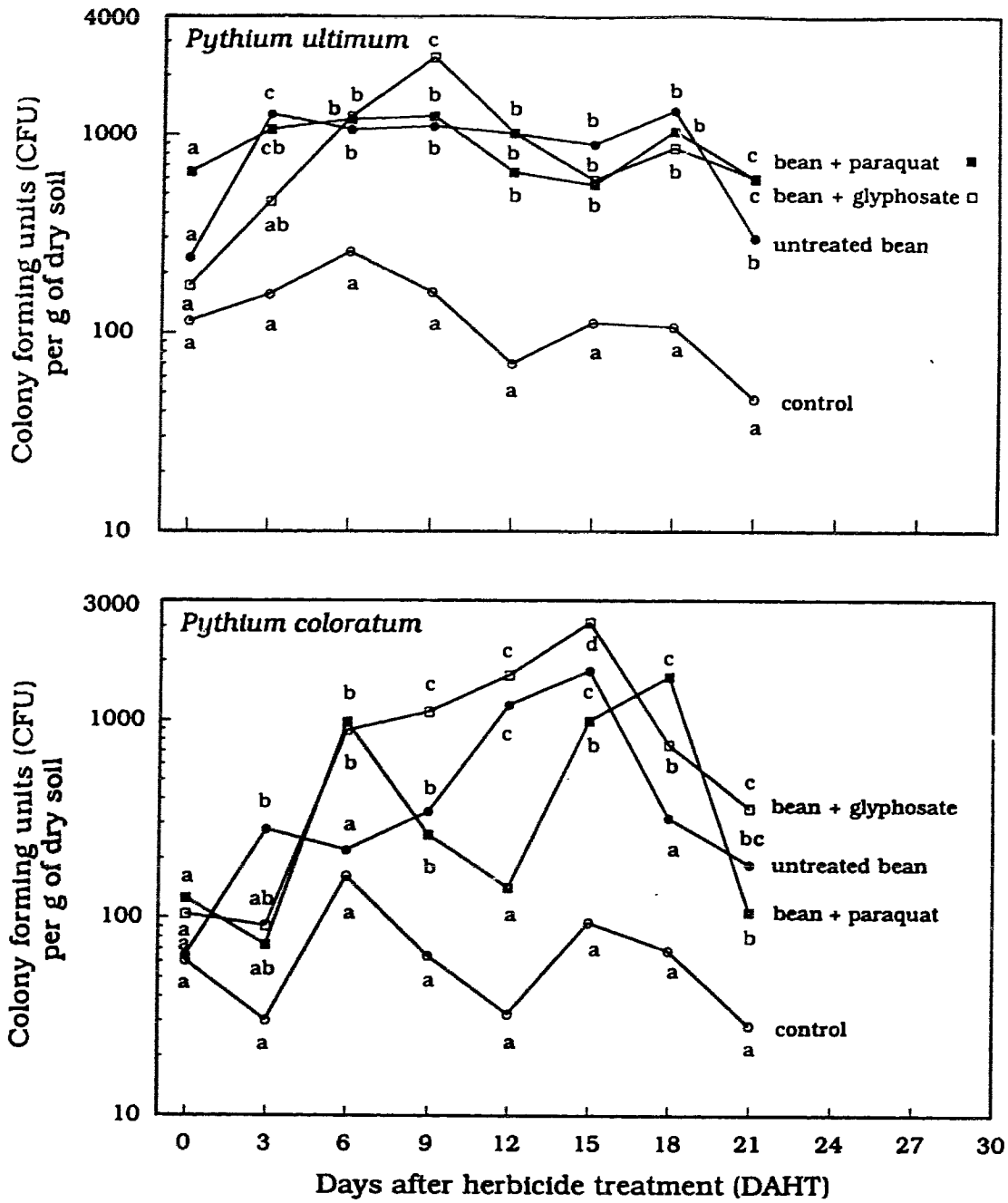
Figure 15. The effect of herbicide-treated bean plants on the population dynamics of *Pythium ultimum* and *P. coloratum* inoculated into autoclaved soil. Symbols within the same DAHT followed by the same letter/s are not significan*ly different from each other (P=0.05).

treatments at 0, 3, 6, 9, 12, 15, 18, and 21 DAHT ranged from 98 to 100% for *P. ultimum* and 94 to 98% for *P. coloratum*, with no significant differences among any of the variables (fungal species, treatment, DAHT).

Sunflower was much less sensitive than pepper to damping-off. The *P. ultimum*-inoculated soils from the various treatments produced an overall range of 1 to 32 % pre-emergence damping-off, and except at 0 DAHT, there were significant differences among the various treatments (Figure 16). More damping off occurred in soils from all three treatments involving beans than in the control (plain inoculated soil) at all sampling times between 6 and 12 DAHT. The highest pre-emergence damping-off of sunflower occurred at 9 DAHT in soils from pots planted with glyphosate-treated beans. When the data for the *P. ultimum* soil plating and sunflower damping-off were compared, a positive correlation between the estimated density of CFU and the percent damping-off of sunflower was apparent.

A low (0.6 - 8.8 %) overall range of pre-emergence damping-off was observed when *P. coloratum*-inoculated soils from various treatments were used for sunflower pathogenicity tests (Figure 16). Except at times 0 and 3 DAHT, significant differences were observed among the treatments. The highest pre-emergence damping-off occurred at 15 DAHT on seeds in soils from pots planted with glyphosate-treated beans. A trend similar to that noticed in *P. ultimum*-inoculated soil for increased damping-off in the soils with the higher *P. coloratum* populations was observed.

Separate correlation analyses of relationships between *Pythium* populations and damping-off of sunflower were done for data obtained from each treatment of both *Pythium* isolates (Table 11). Correlation coefficients (r) of individual treatment data ranged from 0.54 to 0.98,
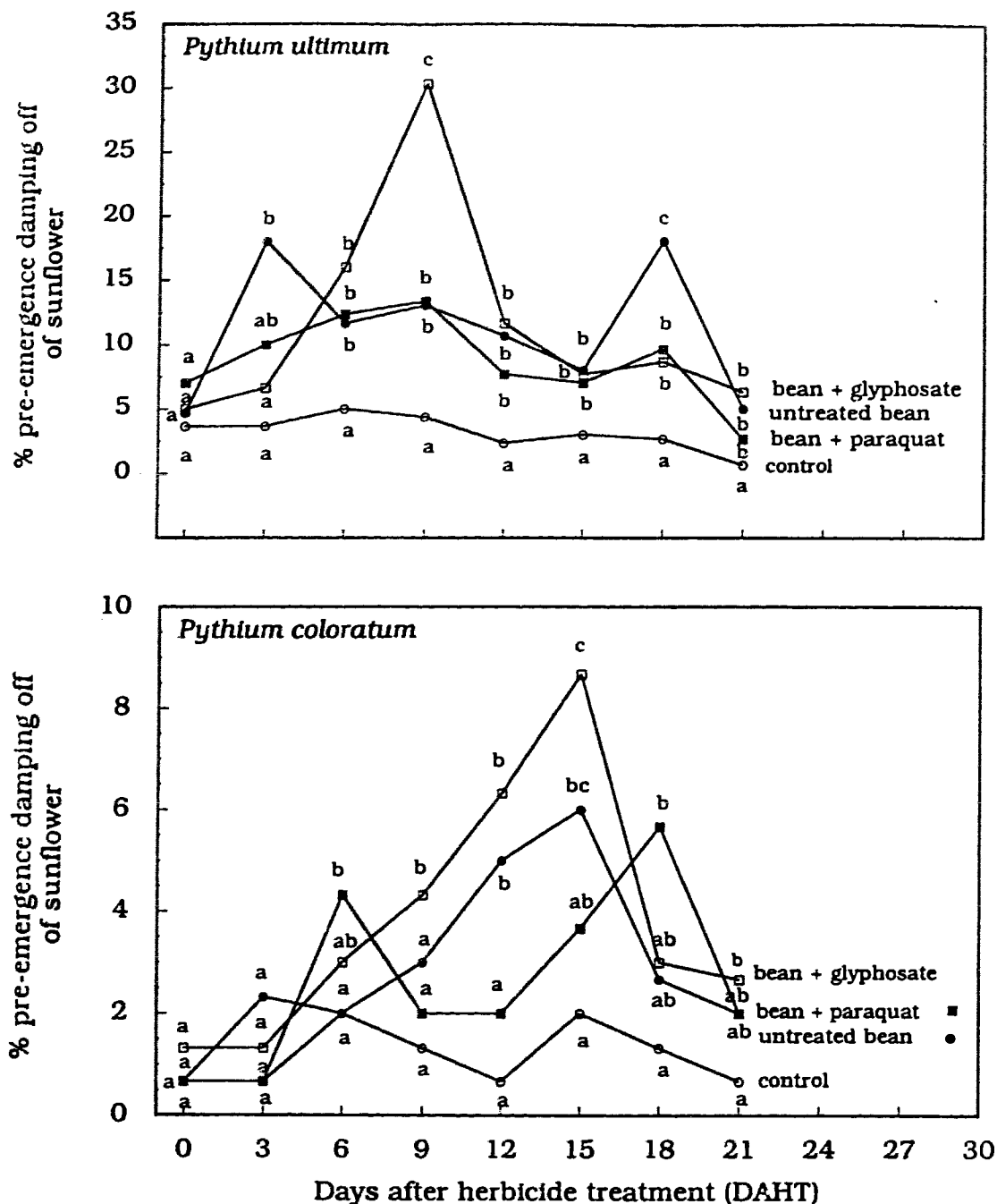
Figure 16. Pre-emergence damping-off observed in germinating sunflower seeds in *Pythium ultimum-* and *P. coloratum-* inoculated soil collected at different DAHT from pots that were used to grow the herbicide-treated bean plants. Symbols within the same DAHT followed by the same letter/s are not significantly different from each other (P=0.05).

Table 11. Summary of correlation analyses for populations of *Pythium ultimum* and *P. coloratum* against the corresponding pre-emergence damping-off observed in germinating sunflower seeds.

Treatments	Correlation coefficient (r)	number of data (n) [a]
P. ultimum		
soil	0.75**	24
soil + bean	0.94**	24
soil + bean + glyphosate	0.98**	24
soil + bean + paraquat	0.64*	24
combined data	0.93**	96
P. coloratum		
soil	0.54*	24
soil + bean	0.77**	24
soil + bean + glyphosate	0.84**	24
soil + bean + paraquat	0.77**	24
combined data	0.92**	96

* significant at P = 0.006
** significant at P = 0.0001

[a] Population levels and % pre-emergence damping-off data obtained at each plating period for each treatment per isolate were combined and correlation test of population vs damping-off were done. Data from all treatments per isolate were also combined and overall correlation of population vs damping-off were determined.

indicating significant correlations between *Pythium* populations and pre-emergence damping-off of sunflower in both *P. ultimum-* and *P. coloratum*-inoculated soils. Overall correlation of pooled data from all treatments for each isolate tested were also significant with r values of 0.93 and 0.92 for *P. ultimum* and *P. coloratum*, respectively.

DISCUSSION

Naturally occurring populations of *Pythium* species, representing undefined components of a complex microflora in a natural soil, and populations of *P. ultimum* and *P. coloratum* that were introduced into sterilized soil were enhanced by the presence of dead and dying bean roots. The *Pythium* populations in both situations began to decline, presumably towards baseline levels, following the observed population increases. The enhanced populations of both GSP and non-GSP in inoculated soils due to glyphosate or paraquat treatment of bean plants growing in these soils resulted in an associated increase in damping off potential of the soil. Thus, the results indicate that root residues in soil from various sources, including those from herbicide-treated plants, can cause temporary increases in *Pythium* populations, and associated increases in damping off potential. The general pattern of sudden *Pythium* population enhancement in both experiments after herbicide treatment of bean plants is consistent with the findings of other researchers who showed root residues from various sources in soil caused temporary elevations of various fungal populations (Lynch and Penn, 1982; Blowes, 1987; Lévesque *et al.*, 1987 and Smiley *et al.*, 1992).

The strong correlations that were observed between the CFU estimates and pre-emergence damping-off of germinating sunflower seeds indicate that the propagules that gave rise to CFU's on the *Pythium*-selective medium were generally capable of infecting germinating seeds.

Sunflower was more resistant to damping-off than was pepper; a similar difference was reported previously (Descalzo *et al.*, 1996b). There were more than 150 *P. ultimum* and 62 *P. coloratum* CFU per g of dry soil in the entire soil plating period. It was apparent that pepper seeds succumb to the damping-off infection even at these lowest *Pythium* population levels in the soil. If the population density of both *Pythium* species had been lower, a difference in pre-emergence damping-off may have been detected.

Although glyphosate treatment of bean plants did not selectively enhance the isolated populations of a GSP over a non-GSP in a simplified soil microflora, differential reponses were observed between the two species in the glyphosate-treated plants. Symptom development and death of the glyphosate-treated plants occurred earlier in *P. ultimum*-than in *P. coloratum*- inoculated soil, and this difference was reflected in the earlier time at which the *P. ultimum* population peaked when compared to *P. coloratum*. Since *P. ultimum* was a fast-growing and more virulent pathogen on 2-week-old bean plants than *P. coloratum* (Descalzo *et al.*, 1996a), the difference in the time of peaking of populations of these two species in pots containing glyphosate-treated bean plants may be attributed to inherent differences in their growth rates and aggressiveness as pathogens.

Another biological difference between the GSP and non-GSP was their saprophytic behaviour in autoclaved soil. *P. coloratum* appeared to

be a more efficient saprophyte than *P. ultimum* since it produced the higher number of CFU during the entire incubation period. Also, the response of the two species to colonization of the previously axenic sandy loam soils by airborne microbial contaminants which were unavoidably introduced when the *Pythium*-inoculated soils were potted at 30 DAI was different. The number of *P. coloratum* CFU was reduced from < 2000 to 62-150 CFU per g of dry soil at this time and for the remainder of the experiment in the controls. In contrast, the axenic population of *P. ultimum* during the same periods did not show any appreciable decline after exposure of the soil to airborne contaminants. This differential response suggests that *P. ultimum* may be more competitive against microbial competition than *P. coloratum.*

The glyphosate LD_{50} for bean seedlings growing in the presence of the GSP and non-GSP isolates used in this experiment was shown to be 0.5 and 50 µg a.i. per plant, respectively (Descalzo *et al.*, 1996a). In the present study, the dose of glyphosate used was comparable to the recommended field application rate, and higher than 50 µg a.i. per plant. This dose would kill bean seedlings directly (without the involvement of GSP), and it is likely that the results described here were at least partially a general response of *Pythium* spp. to the presence of an available nutrient source in the form of dying root biomass of the herbicide-treated plants. A wider differential response might have occurred if a lower glyphosate dose had been used, but the objective of this study was to assess the effect of glyphosate treated plants on population dynamics at simulated field application rates.

Knowing the time and duration of the increases of *Pythium* spp. in soil following glyphosate-treatment of plants is of practical value in

adjusting agricultural practices such as the setting of planting dates, the use of resistant varieties, and crop rotations to avoid the potential pathogenic effects of *Pythium*. This was demonstrated by the results reported by Smiley *et al.*, (1992) who showed that a 2- or 3-week delay in planting of crops after glyphosate treatment, rather than the common practice of planting immediately after spraying, could make a major difference in the severity of *Rhizoctonia* root rot of spring barley. Information on the actual time and duration of population responses of various important soilborne plant pathogens after glyphosate-treatment is currently limited, since it is dependent on numerous parameters, such as soil condition, type of hosts involved, and soil microbial interactions; therefore, more research along this line is clearly needed.

Chapter V

GENERAL DISCUSSION, SUMMARY
AND CONCLUSION

GENERAL DISCUSSION

Colonization of roots of glyphosate-treated plants by *Pythium*

The non-specific nature of GSI involving *Pythium* suggests that the mechanisms by which *Pythium* colonizes the roots of glyphosate-treated plants is similar to the mechanisms by which *Pythium* saprophytically colonizes organic substrates in soil. I propose the following scenario of events to characterize the root colonization process of glyphosate-treated plants by *Pythium*.

It is apparent that roots of plants killed by a lethal dose of glyphosate were readily colonized by *Pythium* as saprophytes. In contrast, there are indications that *Pythium* colonization of roots of plants exposed to a sublethal dose of glyphosate occurred due to glyphosate-mediated disease predisposition in plants. When a glyphosate-containing herbicide is applied in a field situation, individual plants may be exposed to either lethal or sublethal amounts of glyphosate. When plants are exposed to a lethal dose of glyphosate, they are killed directly by the herbicide due to irreversible glyphosate-mediated physiological changes in their normal metabolism. Soilborne *Pythium* may respond to this situation by saprophytic colonization of the dying roots of these plants. However, when plants are exposed to a sublethal dose of glyphosate, they undergo various physiological changes

which are not necessarily lethal (Stasiak *et al.*, 1992). Some of these physiological effects can be observed as phytotoxic symptoms exibited by recovering plants. Other effects of sublethal doses of glyphosate in plants may not be noticeable, such as enhanced root exudation, reduced lignification in root tissues (Liu, 1995) and decreased lignin content of plant tissues (Saltveit, 1988). Enhanced root exudation stimulates *Pythium* propagules within the vicinity of the roots to germinate and start the root colonization process (Campbell and Hendrix, 1973). Reduced lignification of root tissues could favor rapid colonization and spread of *Pythium* inside the tap root and the stem tissues (Liu, 1995). In healthy plants, proliferation and spread of *Pythium* within the main root system is prevented by lignified cell walls, and *Pythium* may remain contained in the root hair zone (Miller *et al.*, 1966). When lignification is reduced by a sublethal dose of glyphosate, *Pythium* contained in the root hairs can resume colonization of the main roots, eventually killing the plant in the process. After the root system of herbicide-treated plants is colonized, *Pythium* produces reproductive structures, such as sporangia and oospores, which remain dormant in the soil until they are stimulated to germinate again to begin another saprophytic or parasitic infection cycle. This may explain the temporary increases in *Pythium* population in soil after herbicide treatment of plants.

Characteristics of *Pythium* as a successful root colonist

Competition among soilborne microorganisms to colonize a potential substrate in soil is intense. *Pythium* spp. in general are weak competitors for substrates that are already colonized by other microorganisms (Barton, 1961; Marx, 1972). To compensate for this,

Pythium spp. utilize four strategies to survive in nature and achieve a strong competitive position in soil. First, *Pythium* has evolved a mechanism to detect the presence of potential substrates by responding quickly to exudates from plant residues and roots, thereby enabling it to be an opportunistic pioneer colonizer of living, dying or dead substrates (Nelson, 1991). Second, *Pythium* rapidly colonizes and assimilates substrates, and converts them into persistent dormant propagules, a phenomenon that Bruel (1975) described as "passive possession of substrates". Conversion of a substrate into numerous finite propagules gives *Pythium* a capability for spatial distribution in soil, which increases its chances of encountering potential hosts. Third, *Pythium* is a well-adapted parasite of plants, it has evolved the ability to infect roots of seedlings and root hairs of mature plants by rapid germination of infective propagules in response to germinating seed volatiles and root exudates in the soil (Nelson, 1987; Miller *et al.*, 1966). Fourth, *Pythium* has developed the capability to parasitize more than one plant species, a characteristic which enhances its opportunities for survival in soil. The evolution of these characteristics enables *Pythium* to successfully compete with other soilborne microflora despite its inability to compete effectively for non-virgin substrates. These characteristics of *Pythium* make it well adapted for rapid root colonization of glyphosate-treated plants.

Interspecific competition among *Pythium* and other soilborne fungi

Interspecific competition among various *Pythium* species may occur when plants are exposed to a sublethal dose of glyphosate. It was observed in Chapter 2 that the fast-growing *Pythium* isolates (*in-vitro*

growth rate) were obtained earlier from roots of glyphosate-treated bean seedlings than were the slower-growing isolates. When the fast- and slow-growing isolates were separately tested for GSI, the results showed that both slow and fast-growing *Pythium* isolates were capable of killing plants treated with sublethal doses of glyphosate 4 weeks after treatment. This result suggests that competition between fast- and slow-growing isolates could occur under natural conditions in the field. Those isolates that penetrate and colonize the root tissues earlier will likely be the more efficient glyphosate synergists in nature. This aspect of GSI was not investigated in this study. Studying the competition among various *Pythium* species in colonizing the roots of glyphosate-treated plants may show the possibility of selective enhancement of the population of aggressive *Pythium* isolates over less aggressive isolates.

The non-specific nature of GSI indicates that other soilborne fungi may also act as glyphosate synergists. There are reported cases of enhanced Rhizoctonia disease in barley that were planted immediately after glyphosate treatment of weeds (Smiley *et al.*, 1992, Roget *et al.*, 1992). Johal and Rahe (1984) isolated several fungi from the roots of glyphosate-treated bean seedlings grown in natural soil aside from *Pythium* spp., and these included *Fusarium spp.*, *Acremonium spp.* and *Trichoderma spp.* The initial root colonization of glyphosate-treated plants by *Pythium* could be followed by secondary invaders. The possible effects of secondary invaders of roots of glyphosate-treated plants in the GSI phenomenon were not examined in this study. Assessment of the occurrence of successions of fungal root colonists of glyphosate-treated plants should be considered in future research.

Potential effect of GSI in increased use of glyphosate

The exclusive authority of Monsanto Corporation to market glyphosate worldwide expired in 1990. As a result, many chemical companies are now able to market a generic herbicide identical to glyphosate. Market competition among various chemical companies will lower the price for glyphosate. The amount of glyphosate used for currently approved purposes is also expected to increase in response to a decrease in price. In Canada, the newly approved application for glyphosate use as a pre-harvest treatment and as a crop desiccant on major cereal grains and oilseeds will also likely increase the use of glyphosate. Although results from my research showed that increases in *Pythium* populations after glyphosate-treatment of plants are likely to be temporary in nature, the compounding effect of repeated glyphosate applications over time is still unknown. It is possible that repeated use of glyphosate in the same area could result in increased *Pythium* populations over time. This aspect of *Pythium* population enhancement is important to consider because the nature and effect of *Pythium* diseases are often overlooked in mature plants, but their effects may be eventually reflected in reduced crop yields. Thus, it is important to determine the effects of GSI in mature herbaceous plants as well as in woody plant species.

Another future situation that guarantees increased usage of glyphosate is in the planting of glyphosate resistant crops. Glyphosate tolerant crops are designed in such a way that glyphosate can be applied in areas where these crops are planted (Mannion, 1995). This type of modern farming technology is almost certain to result in the increased use of glyphosate. There is no information on the resistance of glyphosate

tolerant crops to *Pythium* infection. As mentioned earlier, *Pythium* infection is not easily observed in mature plants, but its effect in decreasing overall yield may be significant. It is, therefore, necessary to determine the effect of *Pythium* infection in glyphosate tolerant crops, and the effect of glyphosate tolerant crops in *Pythium* population dynamics after glyphosate treatment.

Potential research uses of GSI

The potential of using the phenomenon of GSI involving various species of pathogenic *Pythium* as a research tool is increased now that specificity relationships during GSI are defined. The use of seedlings treated with a sublethal dose of glyphosate to selectively isolate the fungal pathogens involved in complex diseases such as apple replant and apple crown rot should be explored.

GSI may also be applied as a method for screening various bacterial and fungal biocontrol agents against important *Pythium* diseases of economic crops. This can be done by testing the potential of candidate biocontrol agents to protect the roots of plant treated with a sublethal dose of glyphosate from colonization by *Pythium.*

SUMMARY AND CONCLUSION

Various *Pythium* species were capable of GSI in various dicot seedlings. These glyphosate synergistic *Pythium* (GSP) were widely distributed in five different soils of varying types and cropping histories. No evidence was found for GSI host specificity among the *Pythium* isolates collected from glyphosate-treated beans and wheat, and among

Pythium isolates from various glyphosate-untreated hosts. The majority of the *Pythium* isolates tested were found to be pathogenic at varying degrees to germinating seeds of various plants.

Inconsistent correlations between glyphosate LD50 values vs *Pythium* growth rates *in-vitro* and virulence indicates the ineffectiveness of using these biological characteristics to predict an isolate's efficacy as a glyphosate synergist.

The general *Pythium* population in natural muck soil and the isolated populations of GSP and non-GSP introduced into sandy loam soil were significantly enhanced by the presence in the soil of bean root residues from various sources. The *Pythium* population increases in both cases occurred immediately after bean root residues became available as substrate (i.e. after foliar application of herbicides or soil amendment with heat-killed bean roots). There were strong positive correlations observed between the population estimates obtained by soil dilution plating and damping-off of sunflower for both GSP (*P. ultimum*) and non-GSP (*P. coloratum*). These results indicate that herbicide treatment of plants can cause at least temporary increases in *Pythium* populations and in the damping-off potential of soils.

In conclusion, the results obtained in this study show that GSI occurs in plants exposed to a sublethal dose of glyphosate. The involvement of *Pythium* species in GSI was attributed to its inherent character as an opportunist and pioneer colonist of virgin, organic substrates in soil. The effects of *Pythium* spp. as glyphosate synergists can be characterized as the initial phase of saprophytic substrate colonization of roots of glyphosate-treated plants.

Future research should consider the effect of interspecific competition between *Pythium* species in GSI. Research to examine the possibility of the role of secondary invaders of roots of plants treated with glyphosate is also recommended. The potential of using the principles of GSI as research tools in isolation of soilborne fungal pathogens of complex diseases, and as a testing protocol for potential biocontrol agents should be investigated.

Literature Cited

Adaskaveg, J.E., Stanghellini, M.E., Gilbertson, R.L. and Egen, N.B. 1988. Comparative protein studies of several *Pythium* species using isoelectric focusing. *Mycologia*, 80: 665-672.

Adegbola, M.O.K. and Hagedorn, D.J. 1969. Host-parasite relations in Pythium bean blight. *Phytopathology*. 59: 1484-1487.

Agnihotri, V.P. 1984. Morphology, survival, and germination of infective propagules of *Pythium* species. Pages 467-487 *in* Mukerji, K.G.,Agnihotri, V.P. and Singh, R.P., eds., *Progress in Microbial Ecology*, Printhouse, India.

Agrios, G.N. 1988. *Plant Pathology*, 3rd ed., Academic Press, Inc., San Diego, California.

Alfaro, A.A. and Mendoza, L. 1990. Four cases of equine bone lesions caused by *Pythium insidiosum*. *Equine Veterinary Journal* 22: 227-228.

Ali-Shtayeh, M.S., Chee len, L. and Dick, M.W. 1986. An improved method and medium for quantitative estimates of populations of *Pythium* species from soil. *Transactions of British Mycological Society* 86: 39-47.

Altman, J. and Campbell, C. 1977. Effect of herbicides on plant diseases. *Annual Review of Phytopathology* 15: 361-385.

Altman, J. and Rovira, A.D. 1989. Herbicide-pathogen interactions in soil-borne root diseases. *Canadian Journal of Plant Pathology* 11: 166-172.

Amrhein, N., Deus, B., Gehrke, P. and Steinrucken H.C. 1980. The site of the inhibition of the shikimate pathway by glyphosate II. Interference of glyphosate with chorismate formation *in vivo* and *in vitro*. *Plant Physiology*. 66: 830-834.

Amrhein, N., Johanning, D., Schab, J. and Schulz, A. 1983. Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. *FEBS Fed. Eur. Biochemistry Soc. Lett.* 157: 191-196.

Ayers, W.A. and Lumsden, R.D. 1975. Factors affecting production and germination of oospores of three *Pythium* species. *Phytopathology* 65: 1094-1100.

Baird, D.D., Upchurch, R.P. and Selleck, E.W., 1971. Phosphonomethyl glycine, a new broad-spectrum, post-emergence herbicide. *Proceedings of Annual California Weed Conference* 24: 94.

Baker, R.J. and Nelder, J.A. 1987. *The GLIM System, Release 3.77, Generalized Linear Interactive Modelling*. Numerical Algorithms Group: Oxford.

Barrett, P.R.F. 1985. Efficacy of glyphosate in the control of acquatic weeds. Pages 365-374 in Grossbard, E. and Atkinson, D., eds., *The Herbicide Glyphosate.* Butterworths, London.

Barton, R. 1961. Saphrophytic activity of *Pythium mamillatum* in soils. II. Factors restricting *P. mamillatum* to pioneer colonization of substrates. *Transactions of British Mycological Society* 44: 105-118.

Benard, D.A. 1994. Role of *Pythium* spp. in the development of cavity spot on carrots in the Fraser Valley of British Columbia. Master of Science Thesis. Simon Fraser University, Burnaby, B.C., Canada.

Biesbrock, J.A. and Hendrix, F.F. Jr. 1970. Influence of soil and temperature on root necrosis of peach caused by *Pythium* spp. *Phytopathology* 60: 880-882.

Blowes, W.M. 1987. Effect of rye grass residues, knockdown herbicides, and fungicides on the emergence of barley in sandy soils. *Australian Journal Experimental Agriculture* 27: 785-790.

Brammall, R.A. and Higgins, V.J. 1987. The effect of glyphosate on resistance of tomato to Fusarium crown root rot disease and on the formation of host structural defensive barriers. *Canadian Journal of Botany* 66: 1547-1555.

Braun, P.G. 1991. The combination of *Cylindrocarpon lucidum* and *Pythium irregulare* as a possible cause of apple replant disease in Nova Scotia. *Canadian Journal of Plant Pathology* 13: 291-297.

Bruehl, G.W. 1975. Systems and mechanisms of residue possession by pioneer fungal colonists. Pages 77-83 in Bruehl, G.W., ed., *Biology and Control of Soilborne Plant Pathogens.* APS Press, St. Paul, MN.

Burr, T.J. and Stanghellini, M.E. 1973. Propagule nature and density of *Pythium aphanidermatum* in field soil. *Phytopathology* 63: 1499-1501.

Cerkauskas, R.F., Dhingra, O.D. and Sinclair, J.B. 1982. Effect of herbicides on competitive saprophytic colonization by *Macrophomina phaseolina* of soybean stems. *Transactions of the British Mycological Society.* 79: 201-205.

Chakravarty, P., and Chatarpaul, L. 1990 Non-target effect of herbicides: II. The influence of glyphosate on ectomycorrhizal symbiosis of red pine (*Pinus resinosa*) under greenhouse and field conditions. *Pesticide Science* 28: 243-247.

Chen, W. 1992. Restriction fragment lenght polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. *Phytopathology.* 82: 1467-1472.

Chen, W. Hoy, J.W. and Schneider, R.W. 1991. Comparisons of soluble protteins and isozymes for seven *Pythium* species and applications of the biochemical data to *Pythium* systematics. *Mycological Research* 95: 548-555.

Christy, S.L., Karlander, E.P. and Parochetti, J.V. 1981. Effects of glyphosate on the growth rate of *Chlorella. Weed Science* 29: 5-7.

Chun, D. and Lockwood, J.L. 1985. Reductions of *Pythium ultimum, Thielaviopsis basicola,* and *Macrophomina* phaseolina populations in soil associated with ammonia generated from urea. *Plant Disease* 69: 154-158.

Clare, B.G., Flentje, N.T. and Atkinson, M.R. 1968. Electrophoretic patterns of oxidoreductases and other proteins as criteria in fungal taxonomy. *Australian Journal of Biological Science* 21: 275-295.

Cole, D.J., Dodge, A.D. and Caseley, J.C. 1980. Some biochemical effects of glyphosate in plant meristems. *Journal of Experimental Botany* 55: 1665-1674.

Cole, D.J., Dodge, A.D. and Caseley, J.C. 1983. Influence of glyphosate on selected plant processes. *Weed Research* 23: 173-183.

Coggins, J.R. 1989. The shikimate pathway as target for herbicides. Pages 97-112 *in* Dodge, A.D., ed., *Herbicides and plant metabolism.* Soc. for Expt. Biol. Seminar Series 38.

Cole, D. J. 1985. Mode of action of glyphosate- a literature analysis. Pages 48-74 *in* Grossbard, E. and Atkinson, D., eds., *The Herbicide Glyphosate.* Butterworths, London.

Comai, L., Sen, L.C., and Stalker, D.M. 1983. An altered *aroA* gene product confers resistance to the herbicide glyphosate. *Science* 221: 370-371.

Comai, L., Facciotti, D., Hiatt, W.R., Thompson, G., Rose, R. and Stalker, D. 1985. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature* 317: 741-744.

Coupland, D. and Caseley, J.C. 1979. Presence of ^{14}C activity in root exudates and guttation fluid from *Agropyron repens* treated with ^{14}C-labelled glyphosate. *The New Phytologist* 83: 17-22.

Curran, J. Baillie, D.L., and Webster, J.M. 1985. Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology* 90: 137-144.

Curl, E.A. and Harper, J.D. 1990. Fauna-microflora interactions. Pages 369-388 *in* J.M. Lynch, ed., *The Rhizosphere.* John Wiley and Sons Ltd., England.

Davies, W.I.C. and Davies, J. 1981. Varying the time of spraying with paraquat or glyphosate before direct drilling of grass and clover seeds with and without calcium peroxide. *Grass and Forage Science.* 36: 65-69.

Deacon, J.W. 1979. Cellulose decomposition by *Pythium* and its relevance to substrate groups of fungi. *Transactions of the British Mycological Society* 72: 469-477.

Descalzo, R.C., Punja, Z.K., Levesque, C.A. and Rahe, J.E. 1996a. Identification and role of *Pythium* species as glyphosate synergists on bean (*Phaseolus vulgaris* L. cv. Topcrop) grown in different soil. *Mycological Research.* (Accepted for publication).

Descalzo, R.C., Punja, Z.K., Lévesque, C.A. and Rahe, J.E. 1996b. Assessment of host specificity among different species of glyphosate synergistic *Pythium. Mycological Research.* (Accepted for publication).

Dick, M.W. 1990. Keys to *Pythium.* College of Estate Management, Whiteknights, Reading, U.K.

Doliner, L.H. 1991. *Discussion Document: Preharvest application of Glyphosate (Roundup_).* Pesticide Directorate, Agriculture Canada, Ottawa.

Doliner, L.H. and Stewart, M. 1992. *Decision Document: Preharvest use of Glyphosate Herbicide.* Pesticide Directorate, Agriculture Canada, Ottawa.

Domsch, K.H., Gams, W. and Anderson, T.H. 1980. *Compendium of Soil Fungi vol. 1.* Academic Press Ltd., London.

Dube, H.C. and Prabakaran, K. 1989. Cell wall degrading enzymes of *Pythium.* Pages 181-188 *in* Agnihotri, V.P., Singh, N., Chaube, H.S., Singh, U.S. and Dwivedi, T.S. eds., *Perspectives in Phytopathology.* Today and Tommorrow's Printers and Publishers, New Delhi.

Dylewski, D.P. and Miller, C.E. 1983. Systematic and host range studies of *Woronina pythii* Plasmodiophoromycetes and host, *Pythium* species, from axenic culture. *Mycologia* 75: 412-422.

Elad, Y. and Chet, I. 1987. Possible role for competition for nutrients in biocontrol of Pythium damping-off by bacteria. *Phytopathology* 77: 190-195.

Endo, R.M. and Colt, W.M.I. 1974. Anatomy, cytology and physiology of infection by *Pythium. Proceedings of American Phytopathological Society Meeting.* 1: 215-223.

Fillatti, J.J., Kiser, J., Rose, B. and Comai, L. 1987. Effecient transformation of tomato and the introduction and expression of a gene for herbicide tolerance. Pages 199-210 in Nevins, D.J. and Jones, R.A., eds., *Tomato Biotechnology*. Alan R. Liss, Inc. New York.

Gardner, D.E. and Hendrix, F.F. Jr. 1972. Carbon dioxide and oxygen concentrations in relation to survival and saprophytic growth of *Pythium irregulare* and *Pythium vexans* in soil. *Canadian Journal of Botany* 51: 1593-1598.

Glass, R.L. 1987. Adsorption of glyphosate by soils and clay minerals. *Journal of Agriculture and Food Chemistry* 35: 497-500.

Gold, S.E. and Stanghellini, M.E. 1985. Effects of temperature on Pythium root rot of spinach grown under hydroponics conditions. *Phytopathology* 75: 333-337.

Golden, J.K., Powell, W.M. and Hendrix, F.F. Jr. 1972. The influence of storage temperature on recovery of *Pythium* spp. and *Meloidogyne incognita* from field soils. *Phytopathology* 62: 819-823.

Gougler, J.A. and Geiger, G.R. 1981. Uptake and distribution of N-phosphonomethylglycine in sugar beet plants. *Plant Physiology* 68: 668-672.

Gresshoff, P.M. 1979. Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. *Australian Journal of Plant Physiology* 6: 177-185.

Griffin, D.M. 1963. Soil Physical factors and the ecology of fungi. II. Behaviour of *Pythium ultimum* at small soil water suctions. *Transactions of British Mycological Society* 46: 368-372.

Grossbard, E. 1985. Effects of glyphosate on the microflora with reference to the decomposition of treated vegetation and interaction with some plant pathogens. Pages 159-185 in Grossbard, E. and Atkinson, D., eds., *The Herbicide Glyphosate*. Butterworths, London.

Grossbard, E. and Atkinson, D., eds. 1985. *The Herbicide Glyphosate*. Butterworths, London.

Grossbard, E. and Wingfield, G.I. 1978. Effects of paraquat, aminotriazole and glyphosate on cellulose decomposition. *Weed Research* 18, 347-353.

Gurusiddaiah, S., Weller, D.M., Sarkar, A. and Cook, R.J. 1986. Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrobial Agents Chemotheraphy* 29: 488-495

Hadar, Y., Harman, G.E., Taylor, A.G. and Norton, J.M. 1983. Effects of pregerminating of pea and cucumber seeds and seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. *Phytopathology* 73: 1322-1325.

Hancock, J.G. 1981. Longevity of *Pythium ultimum* in moist soils. *Phytopathology* 71: 1033-1037.

Hancock, J.G. and Grimes, D.W. 1990. Colonization of rootlets of alfalfa by species of *Pythium* in relation to soil moisture. *Phytopathology* 80: 1317-1322.

Harris, D. and Grossbard, E. 1979. Effects of the herbicide gramoxone and roundup on *Septoria nodorum. Transactions of British Mycological Society.* 73:27-33.

Hendrix, F.F. Jr. and Campbell, W.A. 1973. *Pythium* as plant pathogens. *Annual Review of Phytopathology* 11:77-98.

Hoagland, R.E. and Duke, S.O. 1982. Biochemical effects of glyphosate [N-(phosphonomethyl)glycine], Pages 175-205 *in* Moreland, D.E., St. John, J.B., and Hess, F.D., eds., *Biochemical Responses Induced by Herbicides.* ACS symposium Series, No. 181.

Hollander, H. and Amrhein, N. 1980. The site of inhibition of the shikimate pathway by glyphosate, I. Inhibition by glyphosate of phenylpropanoid synthesis in buckwheat *(Fagopyrum esculentum* Moench.). *Plant Physiology* 66 823-829.

Holliday, M.J. and Keen, N.T. 1982. The role of phytoalexins in the resistance of of soybean leaves to bacteria: effect of glyphosate on glyceollin accumulation. *Phytopathology* 72: 1470-1474.

Howell, C.R. and Stipanovic, R.D. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotc pyoluteorin. *Phytopathology* 70: 712-715.

Howell, C.R. and Stipanovic, R.D. 1983. Gliovirin, a new antibiotic from *Gliocladium virens* and its role in the biological control of *Pythium ultimum. Canadian Journal of Microbiology* 29: 321-324.

Huang, H.C., Morrison, R.J., Muendel, H.C., Barr, D.J.S., Klassen, G.R. and Buchko, J. 1992. *Pythium* sp. "group G", a form of *Pythium ultimum* causing damping-off of safflower. *Canadian Jou.nal of Plant Pathology* 14: 229-232.

Jaffee, B.A., Abawi, G.S. and Mai, W.F. 1982. Fungi associated with roots of apple seedlings grown in soil from an apple replant site. *Plant Disease* 66: 942-944.

Jaworski, E.G. 1972. Mode of action of N-phosphono- methylglycine: inhibition of aromatic amino acid biosynthesis. *Journal of Agriculture and Food Chemistry* 20: 1195-1198.

Jeffers, S.N., Aldwinckle, H.S., Burr, T.J. and Aneson, P.A.. 1982. *Phytophthora* and *Pythium* species associated with crown rot in New York apple orchards. *Phytopathology* 72: 533-538.

Johal, G.S. and Rahe, J.E. 1984. Effect of soilborne plant pathogenic fungi on the herbicidal action of glyphosate on bean seedlings. *Phytopathology* 74: 950-955.

Johal, G.S. and Rahe, J.E. 1990. Role of phytoalexins in the suppression of resistance of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum* by glyphosate. *Canadian Journal of Plant Pathology* 12: 225-235.

Kao, C.W. and Ko, W.H. 1986. The role of calcium and microorganisms in suppression of cucumber damping-off caused by *Pythium splendens* in Hawaiian soil. *Phytopathology* 76: 221-225.

Keen, N.T., Holliday, M.J. and Yoshikawa, M. 1982. Effects of glyphosate on glyceollin production and the expression of resistance to *Phythophthora megasperma* f.sp. *glycinae* in soybean. *Phytopathology* 72: 1467-1470.

Kitchen, L.M., Witt, W.W. and Rieck, C.E. 1981. Inhibition of chorophyll accumulation by glyphosate. *Weed Science* 29: 513-516.

Koike, H. 1971. Individual and combined effects of *Pythium tardicrescens* and *P. graminicola* on sugarcane: a first report. *Plant Disease Reporter* 55: 766-770.

Kouyeas, V. 1964. An approach to the study of moisture relations of soil fungi. *Plant Soil* 20: 351-363.

Kraft, J.M. and Burke, D.W. 1971. *Pythium ultimum* as a root pathogen of beans and peas in Washington. *Plant Disease Reporter* 55: 1056-1060.

Kraft, J.M. Erwin, D.C., 1967. Stimulation of *Pythium aphanidermatum* by exudation from mungbean seeds. *Phytopathology* 57: 866-868.

Lee, T.T. 1981. Effects of glyphosate on synthesis and degradation of chorophyll in soybean and tobacco cells. *European Weed Science Society* 161-164.

Lee, T.T. 1982. Mode of action of glyphosate in relation to metabolism of indole-3-acetic acid. *Physiologia Plantarum* 54: 289-294.

Lévesque, C.A. and Rahe, J.E. 1992. Herbicide interactions with fungal root pathogens, with special reference to glyphosate. *Annual Review Phytopathology* 30: 579-602.

Lévesque, C.A., Rahe, J.E. and Eaves, D.M. 1987. Effects of glyphosate on *Fusarium* spp.: its influence on root colonization of weeds, propagule density in the soil, and crop emergence. *Canadian Journal of Microbiology* 33: 354-360.

Lévesque, C.A., Rahe, J.E. and Eaves, D.M. 1992. The effect of soil heat treatment and microflora on the efficacy of glyphosate in seedlings. *Weed Research* 32: 363-373.

Lévesque, C.A., Rahe, J.E. and Eaves, D.M. 1993a. Fungal colonization of glyphosate-treated seedlings using a new root plating technique. *Mycological Research* 97(3): 299-306.

Lévesque, C.A., Beckenbach, K., Baille, D.A. and Rahe, J.E. 1993b. Pathogenicity and DNA restriction fragment length polymorphisms of isolates of *Pythium* spp. from glyphosate-treated seedlings. *Mycological Research* 97(3): 307-312.

Lévesque, C.A., Vrain, T.C., and De Boer, S.H. 1994. Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. *Phytopathology* 84: 474-474.

Lifshitz, R., Dapler, M., Elad, Y. and Baker, R. 1984. Hyphal interactions between mycoparasite, *Pythium nunn*, and several soil fungi. *Canadian Journal of Microbiology* 30: 1482-1487.

Liu, L. 1995, Studies of mechanisms of pre-disposition by glyphosate of bean roots (*Phaseolus vulgaris L.*) to colonization by *Pythium* spp., Ph. D. Thesis. Simon Fraser University, Burnaby, B.C. Canada.

Lund-Hoie, K. 1985. Efficacy of glyphosate in forest plantations. Pages 328-338 *in* Grossbard, E. and Atkinson, D., eds., *The Herbicide Glyphosate*. Butterworths, London.

Lynch, J.M. and Penn, D.J. 1982. Damage to cereals caused by decaying weed residues. *Journal Science Food Agriculture* 31: 321-324.

McClure, T.T. and Robbins, W.R. 1942. Resistance of cucumber seedlings to damping off as related to age, season of year, and level of nitrogen nutrition. *Botanical Gazzette* 103: 684-687.

Mannion, A.M. 1995. Agriculture, environment and biotechnology. *Agriculture, Ecosystem and Environment* 53: 31-45.

Martin, F.N. 1991. Selection of DNA probes specific for isolates or species in the genus *Pythium* spp. *Phytopathology* 81: 742-746.

Martin, F.N. and Kistler, H.R. 1990. Species-specific banding patterns of restriction endonuclease-digested mitochondrial DNA from the genus *Pythium. Experimental Mycology* 14: 32-46.

Marx, D.H. 1972. Ectomycorrhizae as biological deterrents to pathogenic root infections. *Annual Review of Phytopathology* 10: 429-454.

Middleton, J.T. 1943. The taxonomy, host range, and geographic distribution of the genus *Pythium*. Memoirs of the Torrey Botanical Club. 20: 1-171.

Mielke, H. 1983. Untersuchchungen zum Gaeumannomyces graminis Befall an Winterweizen unter Berucksichtigung einer quenckenbekampung mit "Roundup". *Gesunde Pflanz* 35: 46-49.

Miller, C.R., Dowler, W.M., Peterson, D.H. and Ashworth, R.P. 1966. Obsevations on the mode of infection of *Pythium ultimum* and *Phytophthora cactorum* on young roots of peach. *Phytopathology* 56: 46-49.

Mircetich, S.M. 1971. The role of Pythium in feeder roots of diseased trees and orchard soils in peach tree decline. *Phytopathology* 61: 46-49.

Nafziger, E.D., Widholm, J.M., Steinrucken, H.C. and Killmer, J.L. 1984. Selection and characterization of a carrot cell line tolerant to glyphosate. *Plant Physiology* 76: 571-574.

Nelson, E.D. 1987. Rapid germination of sporangia of *Pythium species* in response to volatiles from germinating seeds. *Phytopathology* 77: 1108-1112.

Nelson, E.D. 1991. Exudate molecules initiating fungal responses to seeds and roots. Pages 197-209 *in* Keister, D.L. and Cregan, P.B. eds., *The Rhizosphere and Plant Growth*. Kluwer Academic Publishers. Netherlands.

Nemec, S. 1971. Mode of entry by *Pythium perniciosum* into strawberry roots. *Phytopathology* 61, 711-714.

Neter, J., Wasserman, W., and Kutner, M.H. 1990. *Applied Linear Statistical Models: regression, analysis of variance, and experimental designs.* 3rd ed., Erwin, Homewood, Illinois.

Parke, J.L. 1990. Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. *Phytopathology* 80: 1307-1311.

Paulitz, T.C. and Baker, R. 1988. Interactions between *Pythium nunn* and *Pythium ultimum* on bean leaves. *Canadian Journal of Microbiology* 34: 947-951.

Punja, Z.K. 1989. Influence of nitrogen and calcium compounds on development of disease due to *Sclerotium rolfsii*. Pages 75-89 *in* Engelhard, A.W. ed., *Soilborne Plant Pathogens: Management of diseases with macro- and microelements.* APS Press, Minnesota.

Qian, P. and Johnson,L.F. 1987. Chemical and physical soil characteristics related to lysis of oospores of *Pythium ultimum*. *Phytopathology* 77: 1062-1066.

Quinn, J.P. 1993. Interactions of the herbicide glyphosate and glufosinate (phosphinothricin) with the soil microflora. Pages 245-265 *in* Altman, J. 4th, ed., *Pesticide Interactions in Crop Production: beneficial and deleterious effects.* CRC Press, Inc. Florida.

Rahe, J.E., Levesque, C.A., and Johal, G.S. 1990. Synergistic role of Soil fungi in the herbicidal efficacy of glyphosate. Pages 260-275 *in* Hoagland, R.E., ed., *Microbes and Microbial Products as Herbicides.* ACS Symposium Series 439.

Rice, J.A. 1988. *Mathematical Statistics and Data Analysis.* Brooks-Cole: Monterey, CA, U.S.A.

Richardson, J.T., Franz, R.E. and Talbert, R.E. 1979. Reactions of *Euglena gracilis* to fluometuron, MSMA, metribuzin and glyphosate. *Weed Science* 27: 619-624.

Rogers, S.G., Brand, L.A., Holder, S.B., Sharps, E.S. and Brackin, M.J. 1983. Amplification of *aroA* gene from *Escherichia coli* results in tolerance to the herbicide glyphosate. *Applied Environmental Microbiology* 46: 37-43.

Roget, D.K., Venn, N.R., and Rovira, D.A. 1987. Reduction of Rhizoctonia root rot of direct-drilled wheat by short-term chemical fallow. *Australian Journal of Experimental Biology* 27: 425-430.

Roisch, U. and Lingens, F. 1980. The mode of action of the herbicide N-phosphonomethyl-glycine: its effect on the growth and the enzymes of aromatic amino acid biosynthesis in *Escherichia coli. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie,* 361: 1049-1058.

Roncadori, R.W. and McCarter, S.M. 1971. Effect of soil treatment, soil temperature, and plant age on *Pythium* root rot of cotton. *Phytopathology* 62: 373-376.

Roslycky, E.B. 1982. Glyphosate and response of the soil microbiota. *Soil Biology and Biochemistry* 14: 87-92.

Rueppel, M., Brightwell, B., Schaefer, J. and Marvel, J. 1977. Metabolism and degradation of glyphosate in soil. *Journal of Agriculture and Food Chemistry* 25: 517-528.

Saltveit, M.E. Jr. 1988. Postharvest glyphosate application reduces toughening, fiber content, nd lignification of stored asparagus spears. *Journal of the American Society for Horticultural Science* 113: 569-572.

SAS Institute INC. 1985 SASR *User's Guide: Version 5 Edition.* Cary: North Carolina.

Saunders, G. A., Washburn, J.O., Egerter, D.E. and Anderson, J.A. 1988. Pathogenicity of fungi isolated from field-collected larvae of the western treehole mosquito, *Aedes sierrensis* (Diptera: Culicidae). *Journal Invertebrate Pathology* 52: 360-363.

Schulz, A., Sost, D. and Amrhein, N. 1984. Insensitivity of 5-enolpyruvylshikimic acid-3-phosphate synthase to glyphosate confers resistance to this herbicide in a strain of *Aerobacter aerogenes*. *Archives of Microbiology*. 137: 121-123.

Scott, W.W. and O'Brien, A.H. 1962. Aquatic fungi associated with diseased fish and fish eggs. *Progress in Fish Culture* 24: 3-14.

Sharma, V. Adee, E.A. and Pfender, W.F. 1989. Effect of glyphosate herbicide on pseudothecia formation by *Pyrenophora tritici-repentis* in infested wheat straw. *Plant Disease* 73: 647-650.

Singh, R.S. and Singh, U.S. 1984. *Pythium* in host and soil. Pages 229-271 in Mukerji, K.G., Agnihotri, V.P. and Singh, R.P.,eds., *Progress in Microbial Ecology*. Printhouse, India.

Smiley, R.W., Ogg, A.G., Jr., and Cook, R.J. 1992. Influence of glyphosate on Rhizoctonia Root Rot, growth, and yield of Barley. *Plant Disease* 76: 937-942.

Spencer, A. 1973. *Guide to the chemicals used in crop protection*, 6th Ed. Agriculture Canada. Ottawa.

Sprankle, P., Meggitt, W.F. and Penner, D. 1975. Absorption, action and translocation of glyphosate. *Weed Science* 23: 235-240.

Spring, O., Albert, K. and Achim, H. 1982. Three biologically active heliangolides from *Helianthus annuus*. *Phytochemistry*, 2110: 2551-2553.

Stasiak, M.A., Hofstra, G. and Fletcher, R.A. 1992. Physiological changes induced in birtch seedlings by sublethal applications of glyphosate. *Canadian Journal of Forest Research* 22: 812-817.

Stedman, O.J. 1982. The effect of three herbicides on the number of spores of *Rhynchosporium secalis* on barley stubble and volunteer plants. *Annals of Applied Biology* 100: 271-279.

Steinrucken, H.C. and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochemistry and Biophysics Research Communication* 94: 1207-1212.

Steinrucken, H.C. and Amrhein, N. 1984. 5-enolpyruvyl shikimate-3-phosphate synthase of *Klebsiella pnuemoniae*. II. Inhibition by glyphosate N-phosphonomethylglycine. *European Journal of Biochemistry* 143: 351-357.

Terry, P.J. 1985. Efficacy of glyphosate for weed control in the tropics and sub-tropics. Pages 375-401 *in* Grossbard, E. and Atkinson, D., eds., *The Herbicide Glyphosate*. Butterworths, London.

Tesar, M.B. 1984. Establishing alfalfa without autotoxicity. Page 29 *in* Report of the Twenty-ninth Alfalfa Improvement Conference, July 15-20, 1984, Lethbridge, Alberta, Canada.

Utkhede, R.S. and Smith, E.M. 1991. *Phytophthora* and *Pythium* species associated with root rot of young apple trees and their control. *Soil Biology and Biochemistry* 23: 1059-1063.

Van der Plaats-Niterink, A.J. 1981. *Monograph of the genus Pythium.* Studies in Mycology no.21. Centraalbureau voor Schimmelcultures: Baarn, The Netherlands.

Van Rensen, J.J.S. 1974. Effects of N-(phoshonomethyl) glycine on photosynthetic reactions in *Scenedesmus* and in isolated spinach chloroplasts. *Proceedings 3rd International Congess on Photosynthesis, Amsterdam.*

Waterhouse, G. M. 1968. *The genus Pythium Pringsheim.* Commonwealth Institute, Mycological Paper 110.

Whigham, D.K. and Stoller, E.W. 1979. Soybean desiccation by paraquat, glyphosate and ametryn to accelerate harvest. *Agronomy Journal* 71: 630-633.

Zar, J.H. 1984. *Biostatistical Analysis*, 2nd Ed., Printice Hall, Englewood Cliff, New Jersey.