STUDIES OF MECHANISMS OF PREDISPOSITION BY GLYPHOSATE OF BEAN ROOTS (*Phaseolus vulgaris* L.) TO COLONIZATION BY *Pythium* spp.

by

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DEGREE: DOCTOR OF PHILOSOPHY

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STUDIES OF MECHANISMS OF PREDISPOSITION BY GLYPHOSATE OF BEAN ROOTS (PHASEOLUS VULGARIS L.) TO COLONIZATION BY PYTHIUM SPP.

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Title of Thesis/Project/Extended Essay

Studies of mechanisms of predisposition by glyphosate of bean roots (*Phaseolus vulgaris* L.) to colonization by *Pythium* spp.

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Abstract

Several possible mechanisms for the predisposition by glyphosate of bean roots (*Phaseolus vulgaris* L.) to colonization by *Pythium* spp. were investigated. Glyphosate at 0.1 and 1.0 µg per ml from the surfactant - containing formulation Roundup® and the nonsurfactant - containing formulation Accord did not affect mycelial growth of *Pythium ultimum* Trow and *P. sylvaticum* Campbell & Hendrix on water agar and cornmeal agar. One µg per ml of glyphosate from both formulations significantly stimulated germination of sporangia of *P. ultimum*. Germination and growth of germ tubes of *P. ultimum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* were significantly greater in root exudates from bean plants whose primary leaves had been treated with glyphosate than that occurring in exudates from non-treated plants. The phytoalexins kievitone, phaseollinisoflavan and phaseollin were detected in roots of bean seedlings grown in natural soil. Comparison of phytoalexin production by bean roots grown in different media indicated that the accumulation of these phytoalexins was probably induced by soil microorganisms. *P. ultimum* elicited kievitone, phaseollinisoflavan and phaseollin in roots grown in sterilized silica sand, while *P. sylvaticum* induced only kievitone and phaseollin in the same growth medium. Glyphosate did not significantly affect the accumulation of phytoalexins within 3 days. However, by day 5, significantly higher phaseollin was detected in the roots of *Pythium* - inoculated plants treated with glyphosate than in *Pythium* - inoculated plants not treated with glyphosate. Glyphosate application did not significantly affect accumulation or exudation of phytoalexins by bean roots grown in a hydroponic system within 3 days. Glyphosate alone reduced the lignin content

of bean roots grown in a peat - based growth medium 72 h after application. In the hydroponic system, the lignin content of roots was increased significantly when *P. ultimum* or *P. sylvaticum* was inoculated. When glyphosate was applied 2 days prior to *Pythium* - inoculation, deposition of lignin in the bean roots was significantly reduced. These results suggest that predisposition by glyphosate of bean roots to colonization by *Pythium* spp. is not due to suppression of phytoalexin accumulation, but may involve changes in root exudates that enhance germination and growth of pathogen propagules, and suppression of a pathogen-induced lignification response by plant roots.

Dedication

To my parents and my husband for their love and encouragement

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Chapter I

Introduction: Relationships Between Herbicides and Plant Diseases

Herbicides are widely used in modern agriculture to control weedy plant species. Several types of interactions are possible when a herbicide is introduced into the environment. In addition to the desired effects on weeds, herbicides may affect soil properties (Burke, *et al.,* 1987), soil microflora (Chakravarty and Chatarpaul, 1990), and the morphology and physiology of non-target crops (Sensei and Loffredo, 1994) These effects can result in increased or decreased incidence of plant diseases. The interactions between herbicides and plant diseases have been extensively reviewed (Altman and Campbell, 1977; Altman and Rovira, 1989; Altman *et al.*, 1990; Lévesque and Rahe, 1992). Herbicides can affect plant diseases by their effects on pathogens. They may influence the growth and reproduction of pathogens directly, or affect microorganisms antagonistic to the pathogens. Herbicides can also change the incidence of plant diseases by predisposing host plants. The physical structures, biochemical defenses or root exudation of plants can be changed by the application of herbicides. Sometimes, disease incidence has been changed by herbicides. However, the mechanisms of such changes are unknown (Hagedorn and Binning, 1982; Rovira and McDonald 1986; Tu, 1987; Bowman and Sinclair, 1989; Geddens *et al.,* 1990; Cohen *et al.*, 1992).

1.1. Direct effects of herbicides on growth and sporulation of pathogens

Effects of chlorophenoxy (2,4-D, 2,4,5-T, 2,4,5-TP, MCPP) and one benzoic acid (dicamba) herbicides on the severity of leaf spot caused by *Bipolaris sorokiniana* (Sacc.) Shoem in leaves of *Poa pratensis* L. were investigated. The soil-drench application of 2,4-D or the spray and soil-drench applications of 2,4,5-T, MCPP and dicamba increased the percentage of diseased tissue on each leaf (Hodges, 1980). *In vitro* tests showed that the vegetative growth (germ-tubes growth and branching, mycelial growth) of *B. sorokiniana* was stimulated by these herbicides at concentrations of 10^{-12} and 10^{-4} M (Hodges, 1977). When 2,4-D, MCPP and dicamba at a concentration of 10^{-6} M were applied to the soil before inoculation, mycelial growth and sporulation on detached leaves of *P. pratensis* exposed to the herbicides were also stimulated as well (Hodges, 1992).

Herbicides that inhibit mycelial growth and suppress the formation of reproductive organs might be expected to reduce the infection potential of pathogens. The herbicides prometryn, fluometuron, simazine and bromophenoxim at concentrations of 4 to 384 ppm significantly inhibited macroconidial germination, germ-tube elongation, growth and sporulation of *Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) Snyder & Hansen and *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hansen, causal pathogens of cotton and tomato wilts in Egypt (El-Abyad *et al.*, 1983). Further research showed that prometryn at 128 and 256 ppm significantly suppressed

respiration rates, reduced absorption of sugar and nitrate, and inhibited rates of synthesis of carbohydrates and organic nitrogenous compounds by both fungi (El-Abyad *et al.*, 1988).

Root rot of seedlings of Monterey pine, *Pinus radiata* D. Don, caused by *Phytophthora cinnamomi* Rands was reduced after replacement of the herbicides simazine and propazine by chlorthal dimethyl and glyphosate to control weeds in nurseries. Laboratory and greenhouse tests showed that chlorthal dimethyl at concentrations of 0.375 and 0.75 g l^{-1} and glyphosate at concentrations of 1.44 and 2.88 g l^{-1} significantly reduced radial growth of *P. cinnamomi* by approximately 10-20 %. The same concentrations of these two herbicides reduced sporangia production by 50 %. In contrast, propazine at concentrations of 1.1 and 2.2 g l^{-1} and simazine at concentrations of 2.5 and 5 g l^{-1} showed varied fungitoxicity *in vitro* and stimulated the production of sporangia by two to four-fold (Kassaby and Hepworth, 1987).

The herbicides, metolachlor, fluometuron, Cotoran multi (50% fluometuron + 50 % metolachlor), prometryne, trifluralin, dinitramine, pendimethalin and butralin, inhibited mycelial growth of the fungi, *F. oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani* Kühn and *Sclerotium rolfsii* Sacc. in culture. The degree of inhibition was related to increased herbicide concentrations from 1 to 100 µg ml^{-1} (Youssef *et al.*, 1985). The application of these herbicides to soil in greenhouse (1, 3 or 5 kg per hectare) influenced the incidence of cotton wilt in greenhouse condition differently depending on the herbicide and the pathogen. The herbicides

decreased the number of diseased plants caused by *F. oxysporum* by 10-65 % with the exception of prometryne and trifluralin, which increased wilt incidence by 10-30 %. The application of metolachlor, prometryne and pendimethalin increased the disease incidence of *R. solani* to a different extent. The number of diseased plants caused by *S. rolfsii* was also increased to various degrees following the application of fluometuron and butralin (Youssef *et al.*, 1985).

1.2. Indirect effects of herbicides on pathogens via other microorganisms

Gaeumannomyces graminis (Sacc.) Arx & Olivier var. *tritici* Walker, the causal organism of take-all disease of wheat, is known to survive in soil within the residues of plant tissues it previously colonized as a parasite (Hornby, 1981). Saprophytic survival of the pathogen on straw in non-sterilized soil was enhanced by diquat (0.075 μg a.i. kg^{-1} soil) + paraquat (0.125 μg kg^{-1} soil) in combination or by glyphosate (0.54 μg kg^{-1} soil). The number of straws positive for the pathogen was increased by 36 and 20 %, respectively. Disease severity in unsterilized soil was also increased by 54 and 22.8 % by these two treatments, respectively. In sterile soil, the herbicide did not affect saprophytic survival on straw or pathogenicity of the pathogen (Mekwatanakarn and Sivasithamparam, 1987). This phenomenon may be associated with inhibition by paraquat of saprophytic colonization of straw by *Trichoderma viride* Pers. ex S. F. Gray, a

common antagonist of fungal pathogens (Wilkinson and Lucas, 1969), and by inhibition of bacterial growth by glyphosate (Roslycky, 1982).

Root rot disease caused by *P. cinnamomi* rapidly increased in intensity after 5 years of continuous use of propazine as a pre-emergent herbicide (Marks and Cerra, 1991). Effects of propazine at half the recommended field application rate on soil, rhizosphere and rhizoplane microflora were examined. In soil, the herbicide increased the number of spore forming bacteria by three-fold, but inhibited Gram-positive and -negative bacteria In the rhizosphere and rhizoplane, the inhibitory or stimulatory effect of propazine was inconsistent. All three bacterial types, especially the spore forming bacteria associated with propazine treatment, enhanced sporangial production of the pathogen *in vitro*. The herbicide chlorthal dimethyl, which suppressed the disease, at 937 mg a.i. l^{-1}, reduced the numbers of all three types of bacteria by 90 % in soil (Marks and Cerra, 1991).

Application of the herbicide ethalfluralin, alone or with vernolate, increased the incidence and severity of net blotch disease of peanut pods in which unspecified actinomycetes seem to be involved. The herbicide ethalfluralin at 15 and 30 $\mu l\ l^{-1}$ was not toxic to fungi or actinomycetes, but was moderately toxic to bacteria in culture. Vernolate was not toxic to any of the microorganisms tested. The populations of actinomycetes in soil increased with a concomitant decrease in populations of bacteria. Ethalfluralin may increase the disease by shifting microbial populations toward an increase in actinomycetes at the expense of other organisms (Ben-Yephet *et al.*, 1991).

1.3. Effects of herbicides on pathogen populations in soil

Fruits of pears and apples are decayed during storage due to the soil-borne fungi *Mucor piriformis* A. Fischer, *Botrytis cinerea* Pers.: Fr. and *Penicillium expansum* Link. Michailides and Spotts (1991) studied the effects of several herbicides on the vegetative growth of the pathogens *in vitro* and on the survival of the propagules of these fungi in soil. Diuron in agar at 4-128 $\mu g\ ml^{-1}$ inhibited germination of spores of *B. cinerea* and *M. piriformis*. Paraquat and 2,4-D at 32 $\mu g\ ml^{-1}$ were inhibitory to *B. cinerea* and *P. expansum*. Mycelial growth of *B. cinerea* and *M. piriformis* was reduced by several herbicides at 128 $\mu g\ ml^{-1}$. Survival of these fungal propagules was generally reduced in both autoclaved and non-autoclaved soil amended with herbicides at 32 or 34 $\mu g\ (g\ dry\ soil)^{-1}$. A decrease of the propagules in soil may reduce the contamination of fruits and lower the incidence of decay.

The responses of nine soilborne fungi and five soilborne bacterial pathogens associated with potato production to herbicides used in potato management were evaluated by Leach *et al.* (1991). Dinoseb at concentrations of less than 64 ppm inhibited growth of all soilborne fungi in amended media. Growth of *R. solani* was almost completely prevented. EPTC, dalapon, linuron, paraquat and dinoseb ranging from 1 to 100 ppm inhibited bacterial survival. A significant reduction of total microorganism populations in the top 5 cm of soil was found when dinoseb was applied at 3.36 kg a.i per hectare to field soil in pots. The large reduction of fungal

activity may help to explain the increased severity of the Rhizoctonia disease complex on potato where dinoseb was not used.

Different levels of EPTC alone or with trifluralin and alachlor did not significantly change root rot caused by *F. solani* f. sp. *phaseoli* (Burk.) Snyd. and Hans. and *R. solani* on pinto bean. The soil populations of these two pathogens were not affected (Gilbertson *et al.*, 1987). The herbicides cyanazine, desmedipham, dicamba, EPTC, ethofumesate, pendimethalin, phenmedipham, trifluralin and 2, 4-D amine were applied preplant, preemergence, postemergence and just before final field operations depending on the weed-management option. Minimum, moderate and intensive use of these herbicides did not affect soil population densities of *Fusarium*, *Pythium* and *Rhizoctonia.* The disease incidence on barley, pinto bean, maize and sugarbeet caused by these pathogens was not changed by these herbicides (Ruppel *et al.*, 1988).

1.4. Predisposition of host plants to disease by herbicides

Trifluralin, prometryne, diuron and fluometuron at concentrations of 100 and 200 mg l^{-1} inhibited mycelial growth of *F. oxysporum* f. sp. *vasinfectum in vitro*. Diuron and fluometuron at 200 mg l^{-1} showed a maximum inhibition of 55 %. The herbicides at lower concentrations (1 and 10 mg l^{-1}, respectively) were less effective. However, all four herbicides at concentrations of 6 and 8 $\mu g\ g^{-1}$ soil increased wilt incidence caused by the pathogen in a susceptible cotton variety by 25-30 % in green

house experiments (Youssef and Heitefuss, 1982). Further research showed that these herbicides increased the accumulation of amino acids in root and seed exudates of the susceptible variety (Youssef and Heitefuss, 1983 a). The germination of conidia and chlamydospores of *F. oxysporum* f. sp. *vasinfectum* was higher in Czapek-Dox-agar containing root or seed exudates of the susceptible cotton cultivar than in the agar media with exudates from a resistant cultivar. Treatment of the plants with prometryne or trifluralin at 0.5, 1.5 and 2.5 mg l^{-1} further stimulated the germination of conidia and chlamydospores of *F. oxysporum* f. sp. *vasinfectum* in the exudates of the susceptible cultivar (Youssef and Heitefuss, 1983 b).

The severity of Fusarium root rot of soybean seedlings in greenhouse and field trials was increased by soil-incorporated trifluralin at 50, 100 and 200 % of the recommended application rate for soybean. Effects of the herbicide on growth or reproduction of *F. oxysporum* were not observed. In this research, injury such as swelling and cracks on treated-seedlings, was evident in the absence of the pathogen. The cracks appeared to be favorable sites for invasion of the pathogen because lesions were usually found at these cracks. The results demonstrated that the primary effect of trifluralin is to predispose soybeans to infection by *F. oxysporum*, not to stimulate growth of the pathogen (Carson *et al.*, 1991)

Leaf spot caused by *B. sorokiniana* on *P. pratensis* was reported to be promoted by low sugar levels (Lukens, 1970). Application of the herbicide MCPP to soil (10^{-4} M) reduced sucrose and total sugar amounts in leaves of

P. pratensis. The decreased content of sucrose and total soluble sugars of uninoculated leaves of herbicide-treated plants was significantly correlated with the increased percentage of diseased leaf area on inoculated plants (Madsen and Hodges, 1983)

Smiley and Wilkins (1992) reported that root rot of winter wheat caused by *R. solani* AG-8 and *R. oryzae* Ryker & Gooch was widely spread after application of the herbicide chlorsulfuron. The herbicide (13.2 g per hectare) increased the severity of Rhizoctonia root rot and reduced the development of winter wheat significantly in greenhouse tests and field experiments. The authors proposed that the increase in disease may be due to detrimental effects of the herbicide on water uptake and absorption of copper and zinc by roots. These inhibitory processes could influence the susceptibility of treated plants to *Rhizoctonia* spp.

1.5. Increase in resistance of host plants by herbicides

The dinitroaniline herbicides, nitralin, trifluralin, pendimethalin, dinitramine, benefin, dibutalin and isopropalin at 1 mg kg^{-1} soil increased the resistance of tomato susceptible to *F. oxysporum* f. sp. *lycopersici.* Foliar applications of nitralin (500 and 1000 ppm) and trifluralin (1000 ppm) also reduced the disease incidence (Grinstein *et al.*, 1984). Neither of these two herbicides inhibited linear growth or conidial germination of the pathogen on agar amended with the herbicides at concentrations of up to 50 $\mu g\ ml^{-1}$. However, fungitoxic compounds which were usually found only in resistant plants were induced in the susceptible tomato after herbicide

treatments. As a result, the treated plants became resistant to *F. oxysporum* f. sp. *lycopersici* (Grinstein *et al.*, 1984).

Pretreatment of melon seedlings of a susceptible cultivar with the herbicide dinitramine (0.5 $\mu g\ g^{-1}$ soil) induced resistance to *F. oxysporum* f. sp. *melonis* Snyder & Hansen. The percentage of diseased seedlings decreased from 90 % in nontreated plants to 2 % in treated plants (Cohen *et al.*, 1986). Ethylene concentrations in stems and leaves were found to increase rapidly in the nontreated plants as wilt symptoms developed. However, ethylene was not detected in the treated plants (Cohen *et al.*, 1986). The herbicide dinitramine is known to retard growth and induce other morphological changes in treated plants (Ashton and Crafts, 1981). Interference in plant hormone activity and synthesis of antifungal compounds by this herbicide may also be involved in the disease reduction (Cohen *et al.*, 1987)

Root application of trifluralin and naphthylacetic acid before infection at concentrations sufficient to induce dwarfing delayed expression and development of Fusarium wilt in tomato plants caused by *F. oxysporum* f. sp. *lycopersici.* The herbicides were not inhibitory to the pathogen growing *in vitro.* The induced resistance in the treated plants was associated with ethylene production before infection (Gentile and Bovio, 1986)

Dalapon and prometryn and linuron at concentrations of 5×10^{-5}, 1×10^{-3} and 2×10^{-3} M induced the resistance of a susceptible cotton cultivar

to vascular wilt caused by *Verticillium albo-atrum* Reinke & Berthier. The same concentrations of the herbicides significantly suppressed mycelial growth of the pathogen in solid and liquid culture. Production of the phytoalexin gossypol in roots and stems of cotton plants was greatly increased in herbicide-treated inoculated plants (Awadalla and El-Refai, 1992).

1.6. Herbicide-fungicide interactions

Fungicides used in plant disease control can be found in the same microenvironment as herbicides and could have interactive effects in plant disease development. Two soil-applied herbicides, fluchloralin and alachlor, altered the activity of fungicides against *Pythium butleri* Subramaniam and *R. solani* causing cowpea damping-off. Both herbicides at concentrations of 1 and 10 μl a.i. l^{-1} reduced the toxicity of 2-methoxyethylmercury chloride and propamocarb to the growth of *P. butleri* and of carbendazim to the growth of *R. solani*, but enhanced the toxicity of captafol and quintozene to *P. butleri* and *R. solani in vitro*, respectively. In pot tests, quintozene gave better control of *R. solani* damping-off in soil treated with either herbicide (5 μl a.i. kg^{-1} soil) than in untreated soil. The efficacy of carbendazim was decreased in the similarly treated soil (Kataria and Dodan, 1982).

The herbicide thiobencarb at 7.5 to 10 μg ml^{-1} inhibited mycelial growth of *R. solani* by 60 % in culture and sclerotial formation was also

inhibited. The fungicide mancozeb had a similar effect at 5 μg ml^{-1} when applied alone. When the fungicide mancozeb and the herbicide thiobencarb were applied in combination, synergistic interactions occurred. Mycelial growth, sclerotial formation and germination of sclerotia in culture and in soil were inhibited to a greater degree than when applied alone, depending on the concentrations of each pesticide in combination (Singh and Sethunathan, 1987).

The fungicide cyproconazole, when applied alone, showed very strong fungitoxicity against *R. cerealis* Van der Hoeven and *Pseudocercosporella herpotrichoides* (Fron) Deighton in culture. In contrast, four herbicides, DNOC, dicamba, ioxynil and bromoxyni, exhibited different growth-inhibiting activity. The mixture of cyproconazole with any of the herbicides resulted in synergistic effects on *P. herpotrichoides* and *R. cerealis in vitro*. EC_{90} (the effective concentration resulting in 90 % growth inhibition) values of cyproconazole were reduced to various degrees depending on the combination. Cyproconazole provided much better disease control in pot tests when used with the herbicides than when it was applied alone. EC_{90} values were reduced from 27 mg l^{-1} to 5-22 ml l^{-1} in mixtures with the herbicides (Kataria and Gisi, 1990)

Interactions between the fungicide chlorothalonil and the herbicide metribuzin toward growth of the fungus *Alternaria solani* Sorauer, the causal agent of early blight on potatoes, have been investigated. *In vitro* tests showed that the type of interactions observed was dependent on the concentrations of the two pesticides. For a given concentration of

chlorothalonil, the herbicide interacted in an additive manner at concentrations ranging from 500 to 900 ppm. In contrast, metribuzin at 1000 ppm acted antagonistically with chlorothalonil (Hill and Stratton, 1991).

The effects of herbicides on plant diseases are summarized in Table 1.1.

1.7. Effects of glyphosate on root diseases of plants

Glyphosate (N-[phosphonomethyl]glycine) is a non-selective, broad spectrum, post-emergence herbicide. It is sold under the trade names of Kleenup, Roundup, Rodeo and Vision (Lévesque and Rahe, 1992). The primary target of glyphosate in plants and microorganisms is an enzyme of the shikimic acid pathway, namely 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which converts shikimate to chorismate (Cole, 1985). By inhibition of EPSP synthase, the biosynthesis of phenylalanine, tyrosine and tryptophan in plants is blocked (Cole, 1985). After application to foliage, it is rapidly translocated to the roots, rhizomes and apical meristems in plants via phloem tissue. This important characteristic results in effectiveness towards perennial weeds. Sprankle *et al.* (1975 a) reported that glyphosate was readily absorbed from a nutrient solution by wheat seedlings. However, only small amounts of glyphosate were absorbed by various plants grown in soil after soil application. These results show that glyphosate is rapidly adsorbed to soil constituents. The slower rate of

Table 1.1. Proposed mechanisms of effects of herbicides on plant diseases

Increased disease	Reduced disease
Stimulation of mycelial growth and sporulation of pathogens	Inhibition of mycelial growth and sporulation of pathogens
Detrimental effects on antagonists of pathogens or beneficial effects on symbiotic microorganisms of pathogens	Reduction of survival of pathogens in soil
Changes in the composition of root exudates	Inhibition of symbiotic microorganisms of pathogens
Physiological and morphological changes of hosts to enhance fungal infection	Induction of antimicrobial compounds in susceptible plants
Antagonistic interactions with fungicides	Synergistic interaction with fungicides

$^{14}CO_2$ evolution from glyphosate in soil containing few microorganisms indicates that glyphosate can be degraded by microflora in soil after binding to soil particles (Sprankle *et al.*, 1975 b). Glyphosate can also be degraded by ultra violet light (Lund-Høie and Firestad, 1986). The preemergence and residual effects of glyphosate in soil are low.

Glyphosate increases the sensitivity of host plants to pathogens. Nonphytotoxic concentrations of glyphosate (4 and 10 $\mu g\ ml^{-1}$) blocked the expression of resistance of soybean hypocotyls inoculated with an incompatible race of *P. megasperma* Drechs. f. sp. *glycinea* T. Kuan & D. C. Erwin (Keen *et al.*, 1982). Roots of tomato seedlings resistant to *F. oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker were colonized by the fungus following exposure to 10 mM of glyphosate for 24 h (Brammall and Higgins, 1988 b). Johal and Rahe (1984) showed that bean seedlings grown in vermiculite or autoclaved soil survived a 10 µg dose of glyphosate while the same dose killed plants grown in natural soil. *Pythium* and *Fusarium* spp. were the predominant colonizers of roots of glyphosate-treated plants. Seedlings grown in autoclaved soil inoculated with *Pythium* spp. were protected from the herbicidal action of glyphosate by a treatment with the systemic fungicide metalaxyl. Subsequent studies demonstrated that *Pythium* and *Fusarium* spp. strongly enhanced the herbicidal efficacy of glyphosate (Johal and Rahe, 1984; Rahe *et al.*, 1990; Lévesque and Rahe, 1992; Lévesque *et al.*, 1992; Lévesque *et al.*, 1993 a, b; Descalso *et al.*, 1995).

1.8. Objectives of the research

Glyphosate at sublethal doses enhances fungal colonization on glyphosate treated plants (Rahe *et al.*, 1990). There is a lack of information on involvement of glyphosate synergistic fungi (GSF) in efficacy of glyphosate on target weeds and non-target woody plants. The effect of glyphosate at sublethal doses on microflora levels and on population of GSF in soil has not been studied. The research on the host specificity of GSF is currently ongoing.

The interactions between GSF and glyphosate treated plants are not well understood. My principal objective was to study the mechanisms by which glyphosate predisposes plant roots to infection by soil-borne fungi, with particular reference to *Pythium* spp., using bean (*Phaseolus vulgaris* L.) as a model plant. The study focused on the effect of glyphosate on both the pathogen and the host plant to determine: (1) direct effects of glyphosate on spore germination and on mycelial growth of *Pythium* spp. and indirect effects on fungal propagules and growth mediated through root exudates; and (2) factors that alter the susceptibility of bean plants, i. e. effects on phytoalexin production and lignification in bean roots.

Since the synergistic effect of glyphosate with surfactant was more rapid but otherwise similar to the effect of glyphosate without surfactant (Johal and Rahe, 1984), the commercial formulation of glyphosate, Roundup® (isopropylamine salt at 359 g l^{-1}) obtained from Monsanto Canada Limited, was used in most of my research presented in this thesis.

The surfactant in Roundup® comprises 15 % of the formulation. It has been referred to as MON 0818. The chemical name of this surfactant is polyoxyethyleneamine. In addition to reducing the surface tension, the surfactant helps the penetration of glyphosate across the leaf epidermal cells. After glyphosate enters a cell, translocation is not affected (L. W. Taylor, Monsanto Canada Inc., *Pers. comm.*). Accord®, a formulation of glyphosate dissolved in water as the isopropylamine salt at 356 g l^{-1} was provided by L. W. Taylor (Monsanto Canada Inc., Delta, British Columbia). Bean seeds used in this research were obtained from Dominion Seed House (Georgetown, Ontario, Canada) or McKenzie Seeds (Brandon, Manitoba, Canada). Metro-mix™, a peat - based growth medium was purchased from W. R. Grace & Co. of Canada Ltd. (Ajax, Ontario, Canada).

Pythium species are prominent among GSF (Lévesque *et al.*, 1993 a, b). The *Pythium* isolates used in this study were selected to represent a fast growing (*P. ultimum* Trow) and a slow growing (*P. sylvaticum* Campbell & Hendrix) synergist; despite the differences in growth rates of the isolates, each was comparable in its synergistic efficacy (Descalzo *et al.*, 1995). In the absence of glyphosate application, the *P. ultimum* isolate was more pathogenic on beans than was the *P. sylvaticum* isolate (Descalzo *et al.*, 1995). In this study, both isolates originating from roots of glyphosate-treated bean seedlings were provided by R. D. Descalzo at Simon Fraser University (Descalzo *et al.,* 1995). They were maintained on sterilized grass leaves in sterilized water until required (Van Der Plaats-Niterink, 1981).

Chapter II

Effect of Glyphosate on Mycelial Growth, Sporangial Germination of *Pythium* spp. and Exudation of Bean Roots

2.1. Introduction

Glyphosate may influence the incidence and severity of plant diseases by affecting fungal pathogens directly. The stimulatory or inhibitory effects of herbicides on various fungi have usually been examined by amendment of media with the active ingredients of herbicides. The commercial formulation of glyphosate, Roundup®, has been reported to inhibit the mycelial growth of many fungi in pure culture (Grossbard, 1985; Kassaby and Hepworth, 1987; Johal and Rahe, 1990). Glyphosate [(isopropyl) ammonium salt] up to 50 $\mu g\ ml^{-1}$ had no effect on growth of *F. graminearum* Schwabe Group 1 on different media (Jeffery and Burgess, 1990). Kawate *et al.* (1992) reported that the mycelial growth and spore germination of *F. solani* f. sp. *pisi* (Jones) Snyd. & Hans. and *P. ultimum* were stimulated or inhibited by glyphosate, depending on concentration. Surfactant - and nonsurfactant - containing formulations of glyphosate at recommended doses were toxic to *Calonectria crotalariae* (C. A. Loos) D. K. Bell & Sobers *in vitro* (Berner *et al.,* 1991).

Various herbicides can affect susceptibility of plants to pathogens by changing root exudates qualitatively and quantitatively (Petersen *et al.,* 1963; Lai and Semeniuk, 1970; Youssef and Heitefuss, 1983 a; Brown and Curl, 1987). Healthy plant roots exude a wide range of organic compounds,

including amino acids, simple sugars, organic acids, vitamins, enzymes and flavonoids. At least ten sugars have been identified in exudates. Glucose and fructose are most abundant. The spectra of amino acids in exudates vary considerably. Twenty-three have been reported in exudates of 15 different species (Schroth *et al.,* 1963; Rovira, 1965). Herbicides can change root or seed exudation indirectly by altering physiological processes or directly by downward translocation of the herbicide molecules and their release from roots into the soil.

Root exudates have received considerable attention because of their potential to influence plant disease development. Positive correlations between susceptibility of plants to pathogens and increases in root exudation have been found (Shao and Christiansen, 1982; Evans and Stephens, 1989; Botha *et al.,* 1990). Root exudates may affect diseases by attracting propagules of pathogens chemotaxically to root surfaces (Morris and Ward, 1992), stimulating germination of propagules (Buxton, 1957; Schroth and Snyder, 1961; Kommedahl, 1966; Mircetich *et al.,* 1968; Chang-Ho, 1970; El-Hamalawi and Erwin, 1986; Chakraborty *et al.,* 1992) and providing nutrients that enhance growth of propagules (Agnihotri and Vaartaja, 1967).

To study the role of glyphosate on predisposition of plant roots to colonization by fungi, three objectives were addressed: (i) to determine the direct effect of glyphosate on germination and growth of glyphosate synergistic isolates of *P. ultimum* and *P. sylvaticum*; (ii) to compare the effect of root exudates collected from glyphosate-treated and non-treated plants on germination of propagules of selected fungi; and (iii) to identify the

effect of glyphosate on the spectrum of amino acids in root exudates of bean, especially on the shikimic acid pathway-related aromatic amino acids, tyrosine, phenylalanine and tryptophan. Preliminary results have been published (Liu *et al.*, 1991).

2.2. Materials and methods

2.2.1. Effect of glyphosate on mycelial growth and sporangial germination of *Pythium* spp. *in vitro*

The surfactant - containing formulation Roundup® and the nonsurfactant - containing formulation Accord® were diluted with distilled water, and 2 ml aliquots containing different amounts of glyphosate were added to autoclaved water agar (WA) and corn meal agar (CMA) to give glyphosate concentrations of 0, 0.1, 1.0, 10.0, and 100.0 $\mu g\ ml^{-1}$ for each formulation. After mixing, 10 ml aliquots of these media were poured into 10-cm Petri plates. After the agar had solidified, each plate was inoculated with a 7-mm diameter plug taken from the margin of 2-day-old *Pythium* colonies grown on CMA and incubated at 22 ^{0}C in the dark. After 24 h, colony radii were measured from three replicate plates of each concentration. Three measurements were taken for each plate. The data from each plate were averaged and then subjected to the Student-Newman-Keuls test for statistical analysis. The experiment was done twice.

To induce sporangial formation by the *Pythium* spp., two agar plugs of *Pythium* culture were placed into a 10-cm Petri plate containing a shallow

layer of water and several 1-2 cm long pieces of autoclaved grass leaf. The water consisted of one part of sterilized pond water (pond located at Simon Fraser University, Burnaby, British Columbia) and one part of sterilized distilled water (PD water) (Van Der Plaats-Niterink, 1981). After 3 to 4 days on the laboratory bench (25 ^{0}C), leaves with sporangia from several Petri plates were transferred to a flask with 15 ml of sterilized PD water and stirred for 1 min. The resulting sporangial suspension was filtered through a layer of cheesecloth and then was centrifuged at 3000 x g for 5 min. The supernatant was discarded and the pelleted sporangia were resuspended in 2 ml of sterilized distilled water. The final concentration of sporangia of *P. ultimum* was adjusted to 2 x 10^3 ml^{-1}.

Fifty µl of sporangial suspension and 50 µl of glyphosate solution diluted from the herbicide formulations as described were added to the wells of cavity slides and mixed. The final glyphosate concentrations in the wells were 0, 0.1, 1.0, 10.0 and 100.0 µg ml^{-1}. Two slides were used for each treatment and were kept at 22 ^{0}C in the dark. After 60 min, sporangial germination was measured. The experiment was done twice.

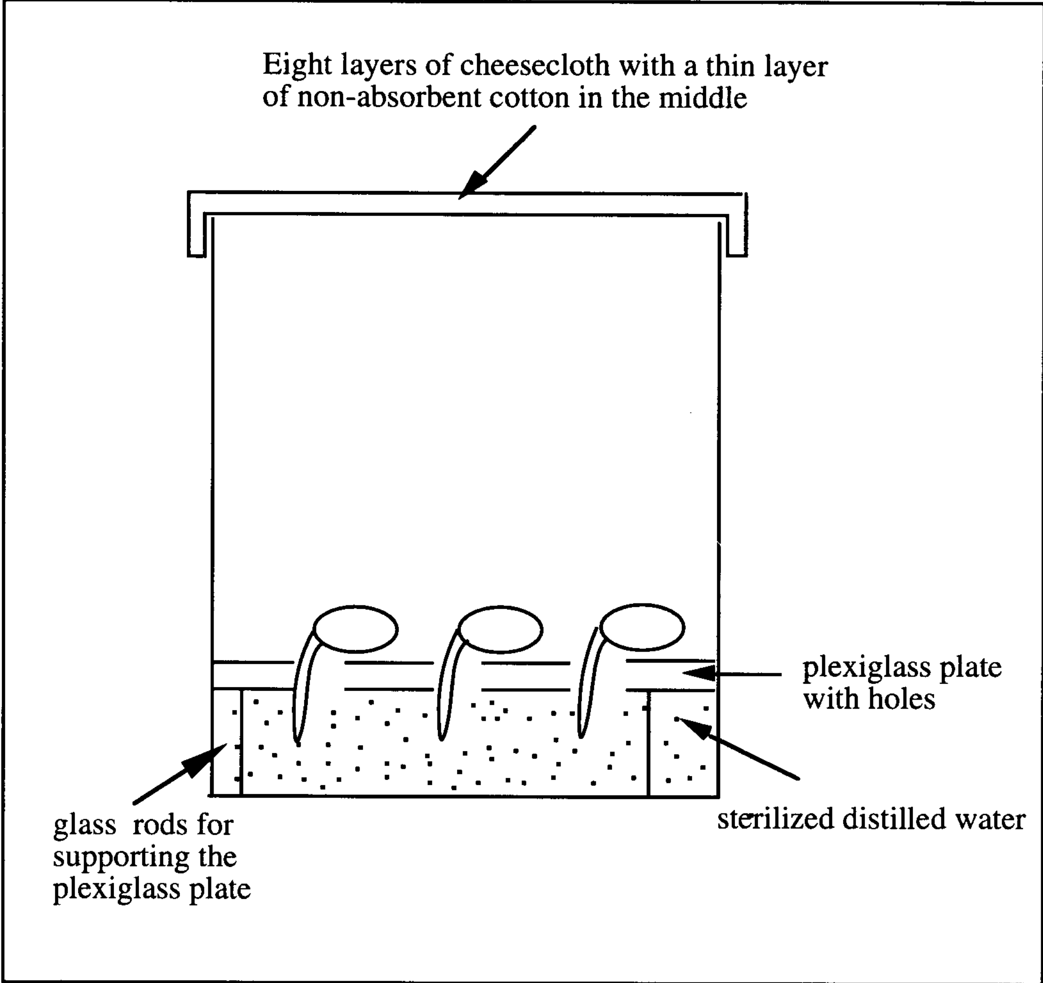
2.2.2. Effect of root exudates from glyphosate-treated plants on germination of sporangia of *Pythium* spp. and microconidia of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)

Bean seeds (cv. Tender Green) were surface sterilized in 1.2% NaOCl for 5 min, washed several times with sterile distilled water and placed on potato dextrose agar (PDA) in Petri plates. After 4 days, clean germinated

seeds with 1-2 cm long radicles were transferred to beakers designed to collect root exudates (Figure 2.1). The beakers, cheesecloth covers and aluminum foil were sterilized by autoclaving. The plexiglass plates were sterilized by soaking in 1.2% NaOCl for 10 min. The plates separated the seeds from the bathing water in the beakers so that exudates from the seeds did not become mixed with exudates from the roots. Cheesecloth covers with a thin layer of non-absorbent cotton in the middle provided air exchange and prevented air-borne contaminants from entering the beakers. Each beaker contained five seeds. The plexiglass plates and the part of the beaker below the plate were wrapped in aluminum foil to keep the roots in the dark. The beakers were placed at room temperature (25 ^{0}C) with a photoperiod of 16 h light and 8 h dark. Light intensity inside the beaker was 28 μmol. m^{-1}. s^{-2}. Each beaker contained 80 ml sterile distilled water which was checked for microbial contamination by streaking onto PDA and nutrient agar (NA) plates 2 days before glyphosate treatment. Only beakers without any evidence of contamination were used in the ensuing experiment.

Five days after transplanting, the seedlings were treated with Roundup® diluted with distilled water to contain 10 μg glyphosate μl^{-1}. The solution was applied to the upper surface of each primary leaf as five 1-μl droplets per leaf so that 100 μg was applied to each seedling. Sterilized distilled water was applied to other seedlings in the same manner as a control treatment. In the first experiment, the sterile water bathing the roots was not changed at the time of glyphosate treatment. In the second experiment, the water bathing the roots was replaced by fresh sterile distilled water just before glyphosate application. Each treatment contained two beakers.

Figure 2.1. A beaker designed for collecting bean root exudates. After 4 days on PDA, the radicles of germinated seeds were inserted through holes in the plexiglass plate so that the roots could develop freely in water.


Eight layers of cheesecloth with a thin layer
of non-absorbent cotton in the middle
plexiglass plate
with holes
glass rods for
supporting the
plexiglass plate
sterilized distilled water

Forty-eight hours after treatment, the aqueous solution bathing the roots in each beaker was collected and concentrated in vacuum at 40 ^{0}C to approximately 4 ml and then transferred to a test tube. The solution was adjusted to 5 ml with sterilized distilled water, passed through a 0.2 μm non pyrogenic filter and stored at -20 ^{0}C before germination assays. The experiment was done twice.

A third experiment examined the early effect of glyphosate on root exudation. Surface sterilized bean seeds were planted into porcelain trays filled with autoclaved Metro-mix™. The trays were watered with distilled water twice a week. After 8 days, the seedlings were carefully lifted and adhering particles were gently washed off under running tap water. The seedlings were transferred to autoclaved 125 ml Erlenmeyer flasks wrapped in aluminum foil. To each flask, 100 ml of sterilized distilled water was added. After transfer, the mouth of the flasks was plugged with sterile non-absorbent cotton. Each flask contained four seedlings. The roots of the seedlings were immersed in the water and leaves of the seedlings were grown outside the flasks. The plants were kept in a growth chamber at 25 ^{0}C and were exposed to a 14 h photoperiod with a light intensity of 240 μmol m^{-2} s^{-1}. After 2 days, the seedlings were transferred to flasks with fresh sterilized distilled water and treated with glyphosate or water as described above. Flasks with the same amount of sterilized water but without bean seedlings were kept under the same conditions as a blank. Each treatment contained three replicate flasks. At 0 (immediately after glyphosate application), 6, 12 and 24 h after treatment, 1 ml of aqueous solution was taken from each flask and stored immediately in microtubes at

-20 ^{0}C.

Sporangia of *P. ultimum* were induced in PD water as described above. FORL isolated from tomato with crown and root rot was provided by Q. Wang at Simon Fraser University and maintained on sterilized Whatman No. 3 filter paper at 4 ^{0}C (Correll *et al.,* 1986). Microconidia of FORL were collected by flooding the 6-day old culture on PDA with distilled water. Propagules from both species were washed with sterile distilled water and centrifuged before being added to slides. The final concentration of sporangia of *P. ultimum* and spores of FORL was adjusted to 2 x 10^3 and 10^6 ml^{-1}, respectively.

To assess germination of the propagules in response to root exudates from treated or untreated seedlings, cavity microscope slides were used. To each cavity, 50 μl of sporangial suspension and 50 μl of root exudate solution were added. For the third experiment, 50 μl of sterilized distilled water from the flask without seedlings and 50 μl of sporangial suspension were mixed in each cavity as a blank. After 60 min at 22 ^{0}C in the dark, sporangial germination and length of germ tubes were measured. Similarly, 40 μl of exudate solution and 10 μl of suspended microconidia of FORL were added to wells of cavity slides. These slides were kept at 22 ^{0}C in the dark, and germination and length of germ tubes were measured after 10 h. Two slides were used for each beaker and approximately 100 propagules were counted for each slide. The experiment was done twice.

2.2.3. Effect of glyphosate on amino acid components of root exudates

Bean seedlings (cv. Tender Green) were grown and treated with glyphosate in beakers containing 100 ml of sterilized distilled water. After 48 h, the aqueous solution from each beaker was collected and stored at -20 ^{0}C without concentration. From each aqueous solution sample, 1 ml aliquots were removed and transferred to individual glass microtubes. To each sample, 20 µl of methionine sulfone (0.4 mM in 0.1 M HCl) was added as an internal standard. After freeze drying in liquid nitrogen, the residue was dissolved in 20 µl of methanol: sodium acetate: triethylamine (2:2:1 by volume) and dried under vacuum. To the dried residue in each tube, 20 µl of derivatization reagent consisting of methanol, triethylamine, water and phenyl isothiocyanate (7:1:1:1 by volume) was added. After 20 min at room temperature (22 ^{0}C), the solution was evaporated to dryness under vacuum. Prior to HPLC, the residue was diluted with 100 µl sample diluent (710 mg of Na_2HPO_4 in 1 liter of water, titrated to pH 7.40 with 10% H_3PO_4, and the resulting solution was mixed with acetonitrile so that acetonitrile equaled 5% by volume). Finally, 20 µl of diluted solution was analyzed by HPLC.

HPLC was performed at 40 ^{0}C with a Waters 5100 liquid chromatograph at 2000 psi, and a Waters 820 data station with a modified gradient table based on the Operator's Manual (Millipore Corporation, 1984) (Table 2.1.). Solvent A consisted of 2 liters of HPLC-grade water, 38 g sodium acetate trihydrate and 1 ml triethylamine. The pH was adjusted to 6.49 by addition of acetic acid and the solution was filtered through a 0.45 µm Nylon-66 membrane. Solvent B consisted of 400 ml HPLC-grade water

Table 2.1. The gradient table of amino acid analysis for HPLC.

Event	Time (min)	Flow rate (ml per min)	Composition A	B	Curve number*
1	0.0	1.0	100.0	0.0	
2	3.0	1.0	100.0	0.0	6
3	10.0	1.0	95.0	5.0	6
4	15.0	1.0	92.0	8.0	8
5	20.0	1.0	92.0	8.0	8
6	30.0	1.0	88.0	12.0	6
7	40.0	1.0	80.0	20.0	6
8	50.0	1.0	75.0	25.0	6
9	60.0	1.0	70.0	30.0	6
10	65.0	1.0	65.0	35.0	6
11	70.0	1.0	0.0	100.0	6
12	75.0	1.0	100.0	0.0	6
13	87.0	1.0	100.0	0.0	6

*A number from 1 through 11 indicating a curve shape that represents the rate of solvent composition change over time during a gradient separation. Curve 6: linear. Curves 7-11: concave.

and 600 ml acetonitrile. The stationary phase was a 3.9X300 mm (particle size: 4 μm) Pico. Tag™ column (Waters, Division of Millipore). The eluents were monitored at 254 nm. Peaks were identified by comparing retention times with standards.

2.2.4. Statistical analysis

The Student-Newman-Keuls test was used for multiple range test. *t*-Test was used to compare paired-means. When the differences between all possible pairs of means were involved, least-squares means tests were employed to determine if data in different treatments were significantly different. In least-squares means test, possible probability values for hypotheses were given. When repeated experiments produced similar results, the data from the different experiments were combined and analyzed as a single experiment.

2.3. Results

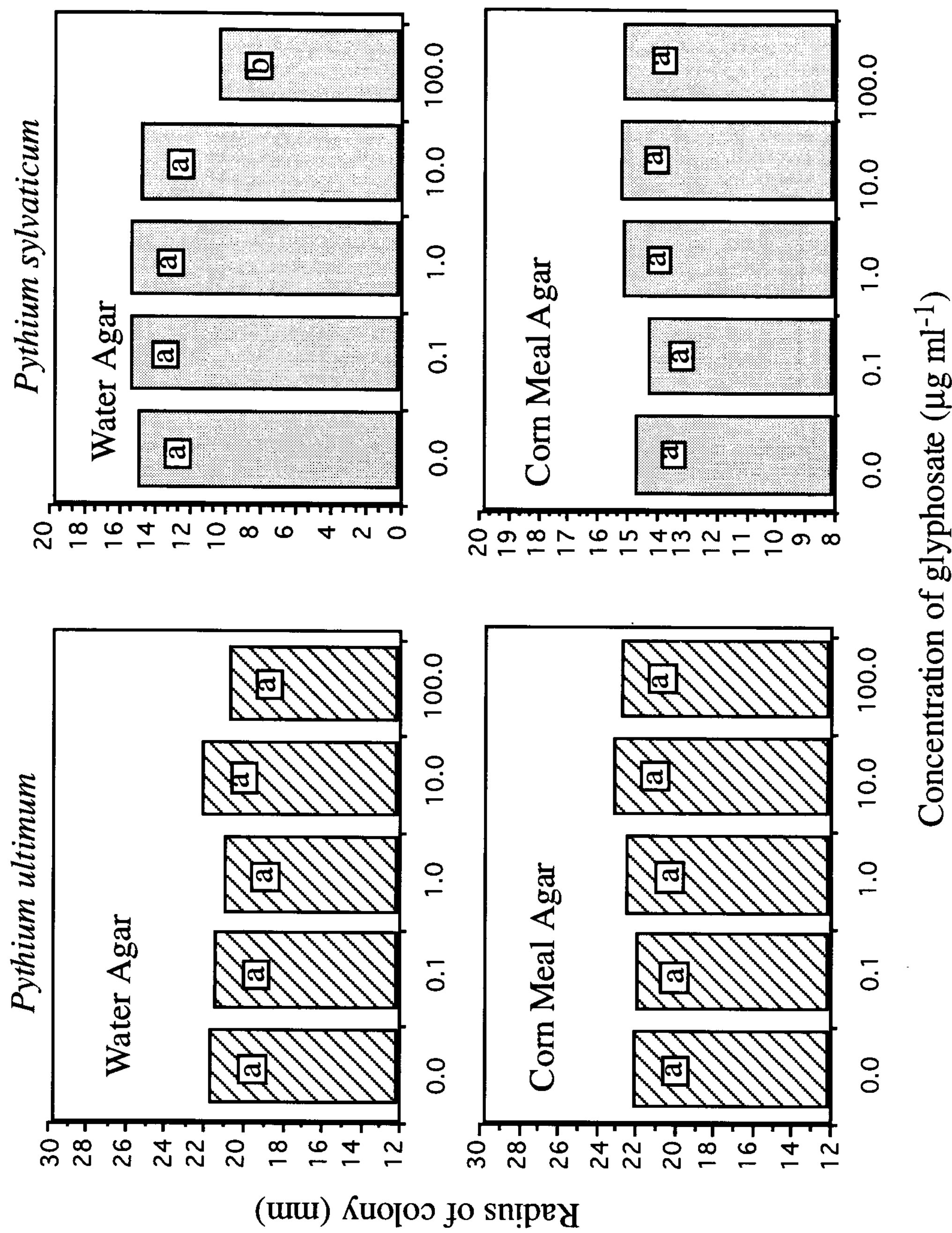
2.3.1. Effect of glyphosate on mycelial growth and germination of sporangia of *Pythium* spp. *in vitro*

The surfactant-free formulation (Accord®) did not affect mycelial growth of *P. ultimum* on either medium at any of the concentrations used. The growth of *P. sylvaticum* on CMA was not affected by Accord®. On WA, Accord® containing 100.0 μg ml^{-1} of glyphosate significantly ($P \leq 0.05$) reduced the growth of *P. sylvaticum* (Figure 2.2). At high concentrations,

the surfactant-containing formulation Roundup® reduced mycelial growth of *P. ultimum* and *P. sylvaticum* significantly ($P \leq 0.05$) on both WA and CMA (Figure 2.3). *P. sylvaticum* appeared to be more sensitive than *P. ultimum* to Roundup® on WA.

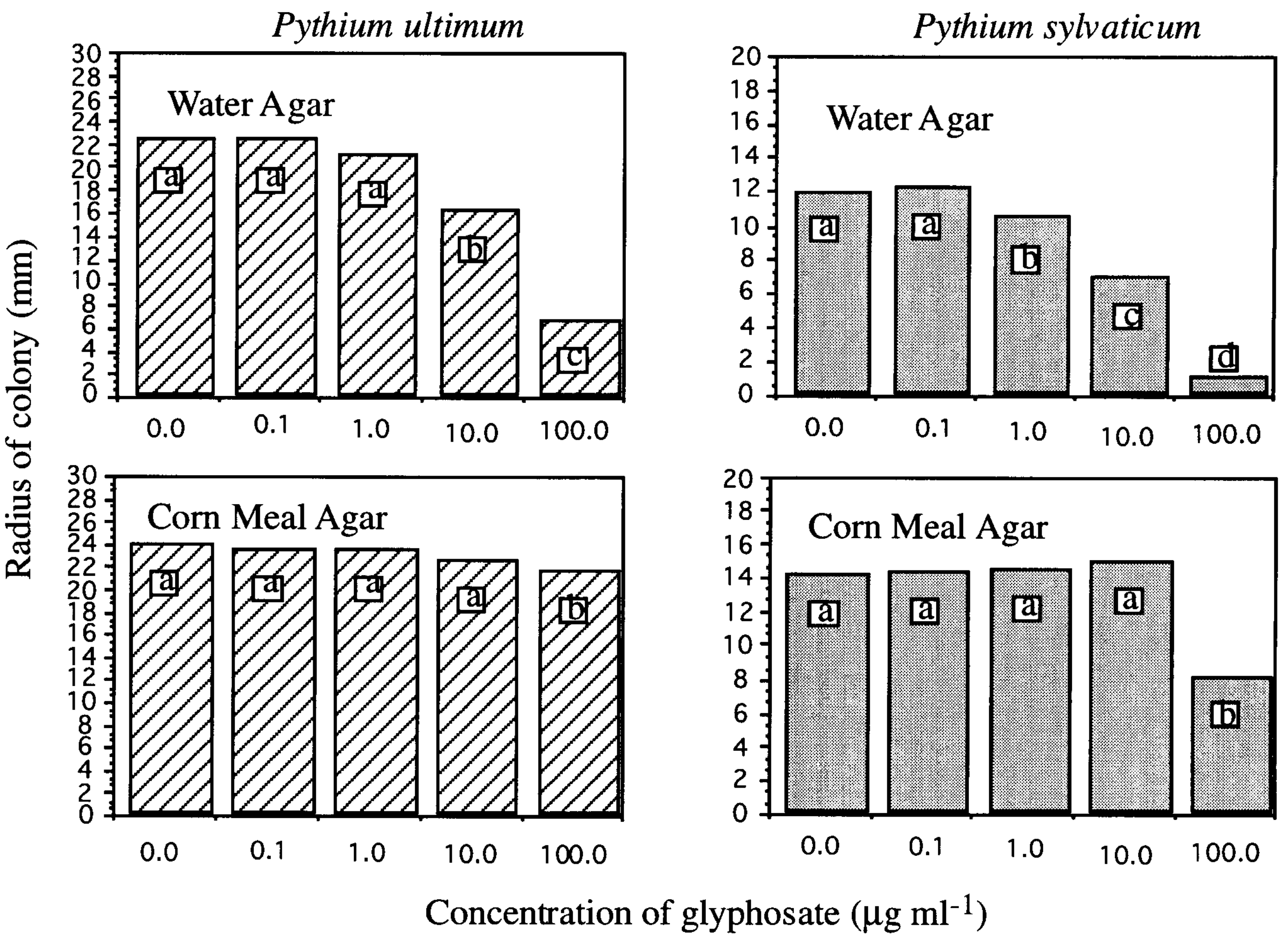
Only *P. ultimum* produced enough sporangia for analysis. Both formulations at concentration of 1.0 $\mu g\ ml^{-1}$ glyphosate stimulated the germination of sporangia of *P. ultimum* (Figure 2.4). Higher and lower concentrations of Accord® did not affect germination (Figure 2.4). Roundup® significantly reduced sporangial germination of *P. ultimum* at 10.0 $\mu g\ ml^{-1}$ glyphosate. At 100.0 $\mu g\ ml^{-1}$, Roundup® completely inhibited sporangial germination (Figure 2.4).

Figure 2.2. Mycelial growth of *Pythium ultimum* and *P. sylvaticum* on agar media amended with the herbicide Accord® (356 g a.i. l^{-1}). The agar plates were kept in the dark for 24 h at 22 ^{0}C. The bars with the same letter are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test (n=6).



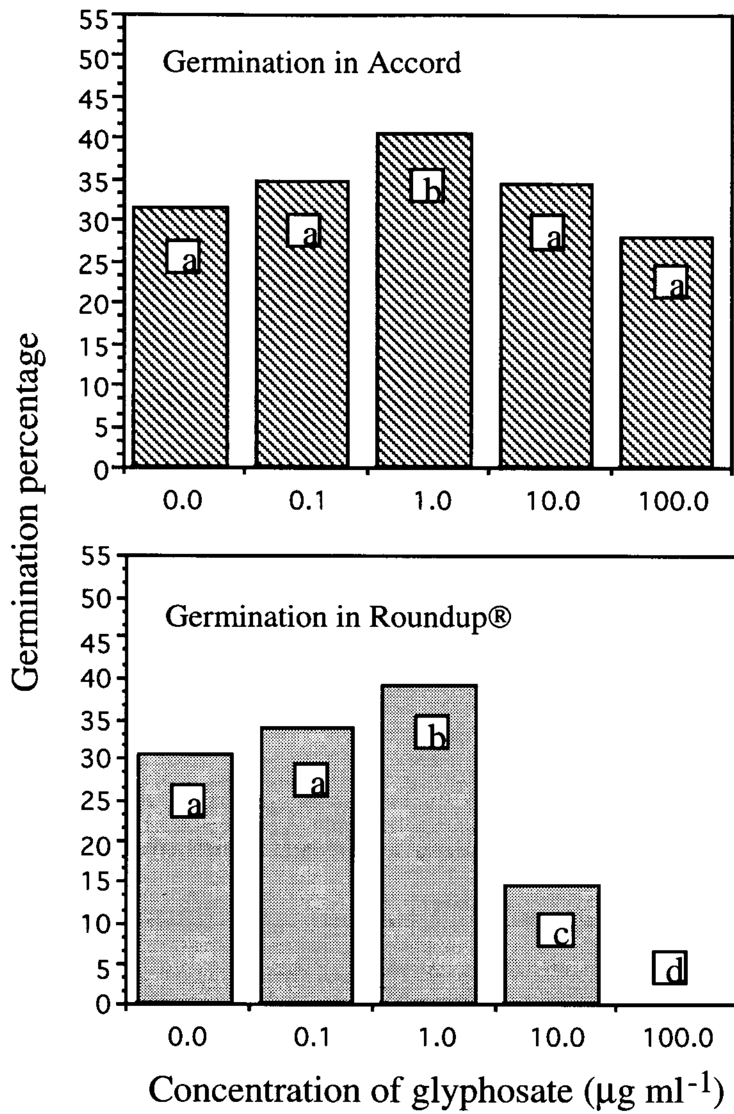
Pythium ultimum
Pythium sylvaticum
Water Agar
Corn Meal Agar
Radius of colony (mm)
Concentration of glyphosate (µg ml-1)
0.0
0.1
1.0
10.0
100.0
a
b

Figure 2.3. Mycelial growth of *Pythium ultimum* and *P. sylvaticum* on agar media amended with the herbicide Roundup® (359 g a.i. l^{-1}). The agar plates were kept in the dark for 24 h at 22 ^{0}C. The bars with the same letter are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test (n=6).



Pythium ultimum
Pythium sylvaticum
Water Agar
Corn Meal Agar
Radius of colony (mm)
Concentration of glyphosate (μg ml-1)
0.0
0.1
1.0
10.0
100.0
a
b
c
d

Figure 2.4. Effect of glyphosate (as Accord® and Roundup®) on germination of sporangia of *Pythium ultimum*. The germination rates were recorded 60 min after incubation at 22 ^{0}C in the dark. The bars with the same letter are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test.


Germination in Accord
Germination percentage
0.0
0.1
1.0
10.0
100.0
a
a
b
a
a
Germination in Roundup®
a
a
b
c
d
Concentration of glyphosate (µg ml-1)

2.3.2. Effect of root exudates from glyphosate-treated bean seedlings on propagule germination

The bean seedlings grew more slowly and developed smaller root systems in beakers compared to their typical development in soil. Otherwise, the plants grown in beakers appeared normal. Root exudates without evidence of bacterial or fungal contaminants were obtained. When the water bathing the roots of bean seedlings was not changed prior to glyphosate treatment, 92.1 and 91.0% of sporangia of *P. ultimum* germinated in control and treatment exudates, respectively. Most microspores of FORL germinated (96.86 % for control and 96.84 % for treatment). For both fungi, germination rates in the exudates from treated and non-treated seedlings were similar. No significant difference was found on length of germ tubes of both fungi in the exudates from treated and non-treated seedlings (Table 2.2.).

When the water bathing the roots was changed prior to glyphosate treatment, the germination rate for *P. ultimum* in the exudates from the treated seedlings was significantly higher ($P \leq 0.05$) than in the exudates from the non-treated seedlings (50.5 % *vs*. 36.0 %). The average length of germ tubes of *P. ultimum* in the exudates from the treated seedlings was significantly higher ($P \leq 0.05$) than that from the non-treated seedlings (Table 2.3). Germination rate for microconidia of FORL in the exudates from the treated-seedlings was significantly higher ($P \leq 0.05$) than that in non-treated seedlings (54.0 % *vs*. 43.1 %). Overall mean length of germ tubes of FORL in the exudates from the treated seedlings was significantly higher ($P \leq 0.05$)

Table 2.2. Mean lengths of germ tubes of *Pythium ultimum* and of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) in root exudates from glyphosate treated and non-treated bean seedlings that had been grown in sterilized distilled water for 5 days prior to treatment.. The primary leaves of seedlings were treated with Roundup®, and the water bathing the roots was collected 48 h after treatment.

Water bathing the roots of seedlings not changed at time of treatment of primary leaves with glyphosate		
	Control	Glyphosate treatment (100 µg per seedling)
P. ultimum [a]	84.8± 2.2	82.6 ± 2.0
FORL [b]	29.9 ± 0.9	29.5 ± 0.9

Water bathing the roots of seedlings not changed at time of treatment of primary leaves with glyphosate		
	Control	Glyphosate treatment (100 µg per seedling)
P. ultimum [a]	44.6 ± 3.7	60.5 ± 3.9*
FORL [b]	15.6 ± 1.5	19.4 ± 1.3*

* significantly different from the corresponding control ($P \leq 0.05$) according to *t*-Test.

[a] Data were recorded after incubation for 60 min at 22 ^{0}C in the dark.

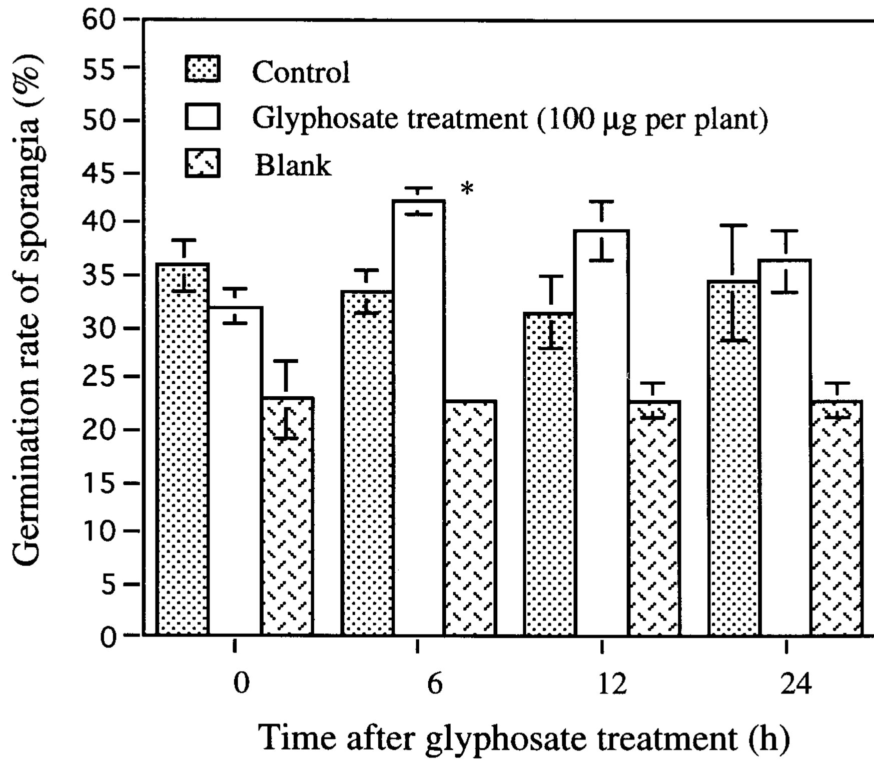
[b] Data were recorded after incubation for 10 h at 22 ^{0}C in the dark.

than that in the exudates from non-treated seedlings (Table 2.2).

In the third experiment, sporangial germination rates of *P. ultimum* during 60 min in distilled water (blank) did not change at any collection times. They remained lower than those in the root exudate, even at 0 h of collection time ($P \leq 0.05$). In this experiment, glyphosate was applied to the primary leaves after the seedlings were transferred to the flasks. Nutrients released from the roots in the period of time (approximately 30 min) used for applying glyphosate may have resulted in the stimulatory effect on sporangial germination at 0 h. Germination rates of sporangia during 60 min in the exudates from the control plants varied at different collection times, but no significant difference was found. In the glyphosate-treated plants, the germination rate in the exudates collected 6 h after treatment was significantly higher than that in the exudates collected at 0 h ($P \leq 0.05$). In the exudates collected at 6 h after glyphosate treatment, the germination rate of sporangia in the exudates from the treated plants was significantly higher than that in control plants ($P \leq 0.01$). At 12 and 24 h, there was no difference in germination rate between control and glyphosate treatment (Figure 2.5).

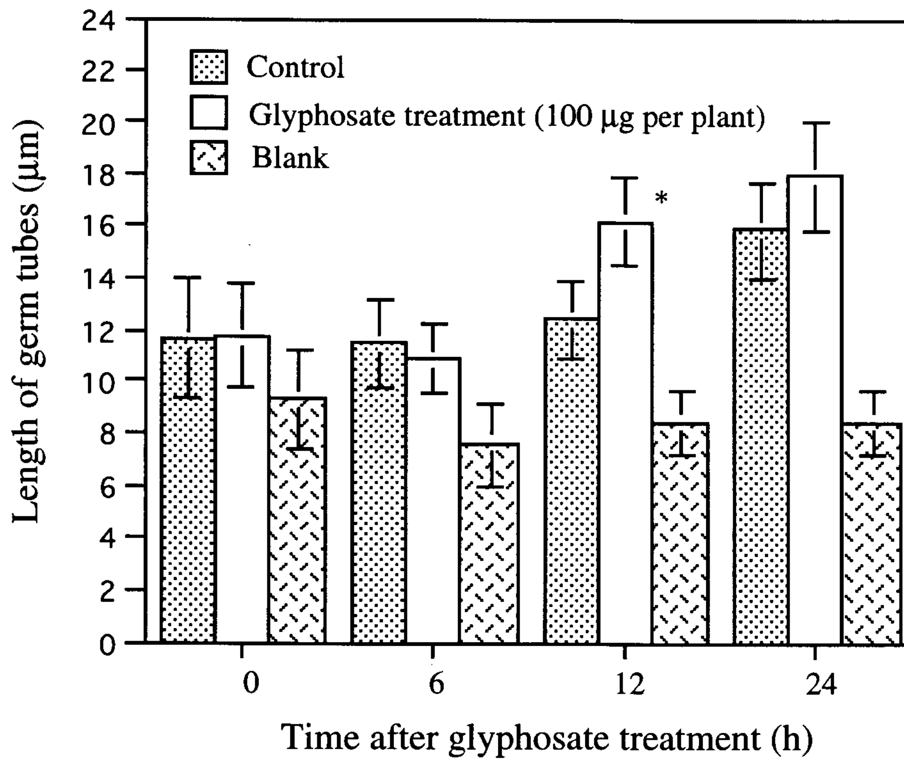
The length of germ tubes produced by germinating sporangia during 60 min in distilled water (blank) and in root exudates from the control collected

Figure 2.5. Germination of sporangia of *Pythium ultimum* in root exudates collected at different times after glyphosate treatment of bean seedlings. Primary leaves of bean seedlings were treated with Roundup®. The germination rates were recorded 60 min after incubation at 22 ^{0}C in the dark. The vertical bars indicate standard errors (n=6). Asterisk indicates significant difference from the control according to least-squares means test ($P \leq 0.01$).


Germination rate of sporangia (%)
60
55
50
45
40
35
30
25
20
15
10
5
0
Control
Glyphosate treatment (100 µg per plant)
Blank
*
0
6
12
24
Time after glyphosate treatment (h)

at different times did not change significantly, while it increased in root exudates from the glyphosate-treated seedlings (Figure 2.6). The length of germ tubes in the root exudates from the glyphosate-treated plants collected 24 h after treatment was significantly longer than those at the exudates collected at 0 and 6 h ($P \leq 0.01$). The lengths of the germ tubes in the aqueous solutions taken from the flasks with the bean seedlings were similar to those incubated in the distilled water at 0 and 6 h of collection time. At 12 and 24 h, the lengths of the germ tubes in root exudates were significantly longer than those in distilled water ($P \leq 0.01$). The marginal difference in length of germ tubes between glyphosate treatment and control was only found in the exudates collected 12 h after glyphosate treatment. At this collection time, sporangia in root exudates from glyphosate treated plants had longer germ tubes than that in exudates from control plants ($P \leq 0.07$) (Figure 2.6).

Figure 2.6. Growth of germ tubes of sporangia of *Pythium ultimum* in root exudates collected at different times after glyphosate treatment of bean seedlings. Primary leaves of bean seedlings were treated with Roundup®. The lengths of the germ tubes were recorded 60 min after incubation at 22 ^{0}C in the dark. The vertical bars indicate standard errors. Asterisk indicates significant difference from the control according to least-squares means test ($P \leq 0.07$).


Length of germ tubes (μm)
24
22
20
18
16
14
12
10
8
6
4
2
0
Control
Glyphosate treatment (100 μg per plant)
Blank
*
0
6
12
24
Time after glyphosate treatment (h)

2.3.3. Effect of glyphosate on amino acid components in root exudates

Aspartic acid, glutamic acid, serine, glycine, α-aminobutyric acid, valine, isoleucine, leucine and phenylalanine were found in exudates from both treated and non-treated beans (Table 2.3). Tyrosine was found, in small but significant amount, only in the exudates from non-treated beans. Serine in the exudates from non-treated beans occurred at a significantly higher concentration than in the treated exudates ($P \leq 0.05$).

2.4. Discussion

My results confirmed the findings that surfactants generally improve the antifungal activity of glyphosate (Grossbard, 1985). For both *Pythium* spp., the formulation Roundup® was more toxic than Accord® and the inhibitory effect was greater on WA than on CMA. Differences in sensitivity of fungi might result from one or a combination of factors. The nutrient content of media may affect the influence of the herbicides on fungal growth (Jeffery and Burgess, 1990). Toxic components in the herbicides may bind with nutrient components in CMA and lose activity. Glyphosate decreases the pH of various media due to its phosphoric acid structure (Kawate *et al.,* 1992). In this study, a decrease of pH (from 6.5 to 5) was observed only in WA with 10 and 100 μg ml^{-1} of glyphosate. The low pH may have contributed to the stronger inhibitory effect of glyphosate in WA.

Glyphosate is rapidly inactivated in soil due to its adsorption by soil constituents (Torstensson, 1985). It is unlikely that the residue concentration

Table 2.3. Amino acids detected in aqueous solution bathing the roots of bean seedlings treated with water (control) or Roundup® (glyphosate at 100 µg per seedling). The data represent accumulation during 7-days of axenic root growth in bathing solution, which includes the 5 days preceeding and 2 days following the time of treatment of leaves with water (control) or glyphosate.

	Concentration (nmol per plant)	
Amino acids	Control	Glyphosate treatment
Tyr	0.98 (0.03)	0.00 (0.00)*
Phe	0.79 (0.49)	2.23 (1.23)
Asp	4.34 (1.84)	3.75 (2.08)
Glu	11.18 (8.28)	11.71 (8.05)
Ser	7.23 (3.24)	2.82 (1.82)*
Gly	7.63 (3.35)	9.87 (2.09)
Aab	2.46 (0.91)	1.79 (0.73)
Val	4.30 (1.35)	5.23 (2.18)
Ile	5.24 (0.99)	6.15 (3.90)
Leu	2.57 (0.54)	10.93 (6.95)
Total	46.72 (15.29)	54.48 (31.11)

Asterisks indicate significant difference from the control according to *t*-Test ($P \leq 0.05$). The numbers in brackets are standard errors (n=4).

of glyphosate in soil would have a significant impact on *Pythium* spp. directly. However, the effect of glyphosate in the rhizosphere is unclear. Rodrigues *et al.* (1982) reported that trace amounts of glyphosate were found in leaves and stems of non-treated corn plants grown in the same pots with wheat seedlings treated with glyphosate (1.1 to 6.7 kg per hectare). The authors concluded that glyphosate was exuded from treated plants and absorbed into roots of adjacent plants. When 10 µl of glyphosate (5 g l^{-1}) was treated to leaves of *Agropyron repens* , glyphosate could be detected in the liquid media surrounding intact roots of the plants 1 day after application (Coupland and Caseley, 1979). A portion of the applied glyphosate in this research may have been exuded from the roots and stimulated germination of *Pythium* spp. in rhizosphere, or the composition of root exudates from the treated plants may have been changed by glyphosate to favor the germination of the pathogens.

The *Pythium* spp. used in this study were able to colonize roots of wheat and bean within 48 h after glyphosate treatment (Lévesque *et al.,* 1993 a). If glyphosate enhanced colonization by stimulating root exudation, stimulation must have occurred within 48 h, which was the time chosen for collection of exudates in this research. Bean seedlings in heat-treated soil survived treatments of 100 µg per plant (Lévesque *et al.,* 1992) and this dose was selected in this study. Sensitivity of sporangia of *P. ultimum* to germination stimulants was reported to be negatively related to endogenous nutrient level (Nelson and Hsu, 1994). In this research, sporangia were cultured on grass leaves rather than on synthetic media to mimic those produced in soil.

When the water bathing the roots was not changed before application of glyphosate, the germination and growth of germ tubes of *P. ultimum* and FORL were not affected. In contrast, root exudates representing only the 2 day post-glyphosate treatment period significantly stimulated both germination and the growth of germ tubes. Several possibilities could account for the different results obtained in the two kinds of root exudates. In the latter case, even though the germination rate was lower unchanged root exudates, the proportion of propagules with long germ tubes was higher. Several reasons may account for these differences. In the case of exudates accumulated over a 7-day period, the roots may have metabolized some exuded stimulants. Alternatively, undetected contaminants could have changed the components of the exudates. Both of these factors would have less of an effect in extracts collected over a 2-day period.

To determine how fast glyphosate could affect root exudation, young seedlings grown in Erlenmeyer flasks were used. The experiment was terminated at 24 h after glyphosate treatment. Any contaminating microorganisms that may have occurred in the bathing solution would likely have had little effect on the composition of the root exudates, especially at the 6 and 12 h sampling times.

Biosynthesis of aromatic amino acids should be decreased by glyphosate according to its mode of action (Cole, 1985). However, the observed effects of glyphosate on the pool of free amino acids including aromatic amino acids in plants are conflicting. Some researchers have reported a decrease in aromatic amino acids and an increase in the total pool

of free amino acids (Nilsson, 1977; Hoagland *et al.,* 1978; Nafziger *et al.,* 1984). Other researchers have reported that glyphosate caused general decreases in free amino acid pools including the aromatic amino acids (Hoagland *et al.,* 1979). Increases in the levels of free amino acids with increases of aromatic amino acids also have been observed (Haderlie *et al.,* 1977; Cooley and Foy, 1992). Jaworski (1972) reported that the amount of free phenylalanine in *Lemna gibba*, an aquatic flowering plant, decreased after the tissue was exposed to glyphosate, while the levels of tyrosine and total amino acids increased.

Water soluble organic materials translocated to roots via phloem can be exuded to the surrounding medium by secretion or leakage. Metabolic energy may be involved in a process of secretion. Secretion usually occurs in the root cap zone and can take place against electrochemical potential and chemical potential gradients (Hale *et al.,* 1978). Leakage, on the other hand, is the loss of compounds in the direction of potential gradients by simple diffusion. The main cause of root exudation is leakage (Hale *et al.,* 1978). Bean roots exude amino acids and sugars into the rhizosphere (Schroth and Snyder, 1961).

When the bathing water was not changed before glyphosate treatment, exudation of tyrosine and serine in treated seedlings was inhibited significantly by glyphosate. The decrease in the level of tyrosine was expected because of the function of glyphosate. Decrease in the level of serine has also been reported in axes of dark-grown soybeans (Hoagland *et al.,* 1979) and carrot cell cultures (Nafziger *et al.,* 1984). Tryptophan was

not found in root exudates of either glyphosate treated or control plants, probably because of low pool size in bean (Mossé and Pernollet, 1983). In carrot cell suspensions, the phenylalanine concentrations decreased soon after treatment but then increased to levels higher than in control suspensions after 3 days (Haderlie *et al.,* 1977). Cooley and Foy (1992) reported that the level of phenylalanine increased in inflated duckweed tissue 48 h after exposure to the chemical. In my results, the concentration of phenylalanine in root exudates from glyphosate-treated seedlings showed an increase, but it was not significantly different from that in root exudates from the control. To my knowledge, the effect of glyphosate on root exudation has not been reported previously. It is possible that concentrations of amino acids in the exudates reflect concentrations of the same compounds in root tissues. However, to determine the effect of changes in amounts of amino acids after glyphosate treatment on germination and growth of sporangia of *P. ultimum*, analysis of root exudates collected within the first few hours after glyphosate treatment needs to be done.

The site of colonization on roots of bean seedlings treated with glyphosate is the region of elongation (Lévesque *et al.,* 1993 a). Rovira (1973) demonstrated that the major zone for release of diffusible exudates is the zone of elongation. My results showed that the root exudates from glyphosate-treated plants had a stimulatory effect on germination and growth of sporangia as early as 6 h after foliar application. Germination of sporangia of soilborne *Pythium* species is a very important step for establishing a host-pathogen relationship. Sporangia germinate only after being stimulated by seed or root exudates (Nelson, 1990). It is possible that

slight changes in root exudates at certain locations of the glyphosate-treated roots can enhance enough colonization by *Pythium* spp. to the roots to cause death of the plants. The changes in root exudation of glyphosate-treated plants may have been an important factor in glyphosate predisposition.

Chapter III

Effect of *Pythium* spp. and Glyphosate on Phytoalexin Production and Exudation by Bean Roots Grown in Different Media

3.1. Introduction

Phytoalexins are low molecular weight, antimicrobial substances produced by plants in response to infection. They appear to be important components of disease resistance mechanisms (Bailey and Deverall, 1971; Rahe, 1973 a; Cruickshank and Smith, 1988). Inhibition of phytoalexin accumulation by treating inoculated plants with inhibitors can affect the outcome of host-pathogen interactions (Keen *et al.,* 1981; Afek and Sztejnberg, 1988). Glyphosate was shown to inhibit phytoalexin accumulation in soybeans, resulting in increased bacterial cell populations in leaves of resistant soybeans (Holliday and Keen, 1982) and to block resistance expression and accumulation of glyceollin in soybean hypocotyls in an incompatible interaction (Keen *et al.,* 1982). The ability of metalaxyl to stimulate glyceollin production in soybean hypocotyls inoculated with a compatible race of *P. megasperma* f. sp. *glycinea* was reduced by treatment with glyphosate (Ward, 1984). Glyphosate also increased the susceptibility of a weed species, *Cassia obtusifolia* L., to a mycoherbicide by suppressing phytoalexin production in the leaves (Sharon *et al.,* 1992). In the bean anthracnose host - parasite interaction, accumulation of phytoalexin was inhibited in incompatible and compatible interactions by sublethal doses of

glyphosate, resulting in occasional 'escape' of hyphae from hypersensitive reactions, and lesions of increased size (Johal and Rahe, 1988; Johal and Rahe, 1990).

The herbicidal action of glyphosate on bean plants in unsterilized soil is due in part to enhanced colonization of the roots of treated plants by fungi, mainly *Pythium* spp. and *Fusarium* spp. (Johal and Rahe, 1984; Rahe *et al.,* 1990; Lévesque and Rahe, 1992; Lévesque *et al.,* 1992; Lévesque *et al.,* 1993 a, b; Descalzo *et al.,* 1995). The major biochemical effect of glyphosate is inhibition of enolpyruvyl shikimate-3-phosphate (EPSP) synthase in higher plants (Cole, 1985). As a result, the biosynthesis of phenylalanine, one of the end products of the shikimic acid pathway, is blocked (Jaworski, 1972; Duke and Hoagland, 1985). Since phenylalanine is involved in the synthesis of isoflavonoid phytoalexins in beans (Bailey, 1982; Smith and Banks, 1986), it is possible that enhanced root colonization associated with glyphosate treatment of beans is due to inhibition of accumulation of phytoalexins in bean roots.

Most studies comparing mechanisms of resistance and susceptibility in beans have been carried out with hypocotyls. Four compounds involved in resistance - phaseollin, phaseollidin, phaseollinisoflavan and kievitone - have been identified (Bailey and Burden, 1973; Smith *et al.,* 1975). It has been reported that some pathogens induce phytoalexin production in root systems (Meyer *et al.,* 1971; Seneviratne and Harborne, 1992). However, conclusions about the role of phytoalexins in disease resistance of roots are contradictory. Some evidence has shown disease resistance to be positively

correlated with early and sufficient accumulation of phytoalexins (Hahn *et al.,* 1985; Bhattacharyya and Ward, 1986; Sulistyowati *et al.,* 1990; Huang and Barker, 1991); in other studies, accumulation of phytoalexins was similar in the roots of both susceptible and resistant cultivars (Keen and Horsch, 1972), and induction of phytoalexins did not prevent pathogenesis (Ibrahim *et al.,* 1982; Ransom *et al.,* 1992).

The accumulation of phytoalexins in bean roots occurred in response to various chemical treatments and to a fungal cell wall elicitor (Goossens *et al.,* 1987). The relationship between phytoalexin production in bean roots and infection by microorganisms has not yet been explored. The objectives of this research were to (i) characterize phytoalexin production by bean roots qualitatively and quantitatively; (ii) determine if phytoalexin production in roots was constitutive or inducible; and (iii) determine the effects of sublethal doses of glyphosate on phytoalexin accumulation in root tissue and secretion of phytoalexins into the surrounding growth medium. Preliminary findings have been published (Liu *et al.,* 1992; Liu *et al.,* 1994).

3.2. Materials and Methods

3.2.1. Phytoalexin production by roots of bean seedlings grown in various media

The initial experiments involved non-sterilized seeds (cv. Tender Green) planted individually in Styrofoam coffee cups containing 150 ml of

non-sterilized soil. Seedlings were harvested 7 days after seeding. The second set of experiments involved five bean cultivars: Dandy, Dark Red Kidney, Eastern Butter Wax, Goldcrop Wax and Tender Green. Non-sterilized seeds were planted individually in Styrofoam cups containing 150 ml of non-sterilized loamy sand soil collected at a garden plot at Simon Fraser University (Burnaby, British Columbia, Canada). The roots were harvested for analysis 12 days after seeding. Unless specified otherwise, the cultivar Tender Green was used in all subsequent experiments. The third set of experiments compared phytoalexin production by roots of seedlings grown in either 0.65% water agar (Anachemia Science, Division of Anachemia Canada Inc., Montréal, Canada), silica sand (Imasco Mineral Inc., Surrey, British Columbia, Canada), Metro-mix™, sterilized loamy sand soil or non-sterilized loamy sand soil described in the second set of experiments. Magenta™ (Sigma Chemical Co.) boxes (6 x 6 x 20 cm) containing 100 ml of water agar or 125 g silica sand were sterilized by autoclaving at 121 ^{0}C for 15 min. Metro-mix™ in plastic bags was autoclaved in the same way and transferred to open 150 ml Styrofoam cups. The seeds were surface sterilized in 1.2% NaOCl for 5 min, rinsed three times with sterile distilled water and then were planted individually into these containers. Glass beakers (1000 ml) covered with cheesecloth were used for the experiment with the sterilized soil. Each beaker contained 200 g moist soil and was autoclaved twice on 2 consecutive days. Seeds were surface sterilized and planted into the beakers individually 3 days after the second sterilization. Non-sterilized seeds were planted into the natural soil as described before. All growth media except water agar were kept moist by addition of sterilized distilled water as required.

Seedlings were grown at 22 ^{0}C under a 14 h photoperiod with 240 µmol m^{-2} s^{-1} light intensity. The roots in the different media except in water agar were collected 10 days after seeding. The plants in water agar reached the height of the containers (20 cm) in 7 days, and the roots of the seedlings were harvested at that time.

3.2.2. Extraction and analysis of phytoalexins

Root tissue was excised from the shoot tissue at the point of emergence of the first laterals, cut into 0.5-1 cm pieces and extracted with 95% ethanol (Rahe, 1973 a) except that the final residues were dissolved in 400 µl of 95% ethanol. For the first set of experiments, hypocotyls below the soil surface were cut and extracted the same way as roots. Individual samples consisted of roots or hypocotyls from three or four plants (to make up 1.0 g fresh tissue). Two replicate samples were made for each treatment in each experiment, and the experiments were done twice

Qualitative analysis of phytoalexins was done by TLC and spectrophotometry. Forty µl aliquots of each sample were applied to thin layer of silica gel on 20 x 20 cm plates (silica gel 60, thickness 0.2 mm, BDH Chemicals). Ten µg of authentic phaseollin, phaseollinisoflavan (PIF) and kievitone (authentic samples kindly provided by Dr. D. A. Smith, University of Kentucky, Lexington, KY, U.S.A.) were applied separately as standards. Chromatograms were developed with benzene:ethyl acetate:formic acid (74/25/1 v/v/v) and examined under ultraviolet light (UV) of 354 nm. UV-Absorbing zones in the sample lanes with the same

Rf as the authentic phytoalexins were scraped from the TLC plates and extracted three times with 10 ml volumes of 95% ethanol. The combined extracts were taken to dryness under vacuum at 40 ^{0}C, and the residues were dissolved in 100 μl of 95% ethanol. UV absorption spectra of the samples were obtained with a 3000 Array Spectrophotometer (Milton Roy Company, Analytical products division, Rochester, NY, U.S.A.) and compared with those of the authentic standards.

Quantitative analysis of phytoalexins was done using HPLC on a Maxima 820 Chromatography Workstation (Waters, Division of Millipore, Milford, Massachusetts, U.S.A). A 50 x 4.6 mm guard column and a 250 x 4.6 mm C18 10 μm Spherisorb™ column (Phenomenex, Torrance, CA, U.S.A) were maintained at 30 ^{0}C and eluted with a mobile phase composed of two solvents: (A) 33.5% acetonitrile, 66.5% HPLC grade water, and (B) 81% acetonitrile, 19% HPLC grade water. The elution program started at 100A:00B for 5 min, followed by a linear gradient to 00A:100B over the next 5 min. Eluent composition was then maintained at 00A:100B for an additional 5 min. The original column conditions were restored by a linear gradient over 10 min and kept at the starting condition for 5 min before each run. A constant flow rate of 1.0 ml min^{-1} was maintained. Samples were monitored at 280 nm. Retention times under these conditions were typically 13.7, 14.3 and 15.1 min for kievitone, PIF and phaseollin, respectively. The identity of peaks in the samples was verified by comparing mass spectra of samples with the spectra in published papers (Burden *et al.,* 1972; Smith *et al.,* 1973 b) and spectra of the phytoalexin standards. The amounts of phytoalexins in the samples were estimated by

reference to standard curves prepared by HPLC analysis of authentic phytoalexins.

3.2.3. Effect of glyphosate application and inoculation with *Pythium* spp. on phytoalexin production by bean roots grown in silica sand

Glyphosate synergistic isolates of *P. ultimum* and *P. sylvaticum* were maintained on sterilized grass leaves in sterilized water in the dark at 22 ^{0}C until required as described in Chapter II.

Surface-sterilized bean seeds were planted individually in silica sand in Magenta™ boxes as described previously. After 7 days, 100 µg of glyphosate was applied to each seedling as five 1 µl droplets of diluted Roundup® on each primary leaf. Immediately after glyphosate treatment, four 7 mm-diameter agar plugs from 2-day old cultures of *P. ultimum* or *P. sylvaticum* on potato dextrose agar (PDA) were placed, one at each corner of the box, and covered with sand. Other treatments received either the same dose of glyphosate alone or were inoculated with *Pythium* alone. Plants that received neither glyphosate nor *Pythium* spp. served as control. The plants were kept under the conditions described previously. The presence of contaminants in the sand was checked on PDA and NA plates 3 days before glyphosate treatment. Only plants in sand lacking evidence of microbial contamination were used in further analysis. At 0, 1, 3 and 5 days after glyphosate treatment, six plants from each treatment were randomly collected and the fresh weight of the roots comprising each replicate was

measured. The tissue was extracted for phytoalexins as described. Each replicate comprised the roots from three plants, and the experiment was done twice.

3.2.4. Determination of time of inoculation with *Pythium* spp. and time of phytoalexin collection in a hydroponic system

The primary goal was to study effects of glyphosate on accumulation of phytoalexins in roots and its effect on phytoalexin exudation into the growth medium. During the research, it was found that extracts from some batches of clean silica sand purchased from the same supplier had very strong absorbance under UV light, which interfered with the quantitative analysis. As a consequence, a hydroponic system was developed for subsequent investigations.

Surface-sterilized bean seeds were planted into porcelain trays filled with Metro-mix™ which was autoclaved twice for 30 min at 24 h intervals and left at room temperature (22 ^{0}C) for 3 days before use. The trays were watered with distilled water twice a week. After 8 days, the seedlings were carefully lifted and adhering particles were gently washed off under running tap water. The seedlings were transferred to autoclaved 20 ml glass vials containing 18 ml of sterilized one-tenth strength Hoagland's nutrient solution (Tuite, 1969). The vials were then plugged with pieces of autoclaved non-absorbent cotton and assigned randomly to test tube racks which were wrapped with aluminum foil to keep the roots in the dark. The

racks were kept at 25 ^{0}C and a 14 h photoperiod with a light intensity of 240 μmol m^{-2} s^{-1}. The nutrient solution was replenished to 18 ml daily.

P. ultimum and *P. sylvaticum* were cultured in V8 liquid medium (Ayer and Lumsden, 1975). After 8 days, mycelial mats were collected, blotted to remove free water, and homogenized in 0.08% water agar (1/100 w/v for *P. ultimum* and 2/100 w/v for *P. sylvaticum*) in an Omni-Mixer (Ivan Sorvall, Inc. Norwalk, CT. U.S.A) at speed 5 for 20 seconds. To confirm glyphosate synergism in the hydroponic system, different doses of *Pythium* inoculum and glyphosate were used. For *P. ultimum*, 0.5, 1 or 2 ml of the mycelial suspension were prepared as above and added to each vial. For *P. sylvaticum*, 1 or 2 ml was added. Control seedlings without *Pythium* received 2 ml of 0.08 % water agar. Immediately after inoculation, 0, 25 or 50 μg glyphosate was applied to the primary leaves as ten 1 μl droplets of water or an appropriate dilution of Roundup®. To determine the herbicidal effect, 50 μg glyphosate was applied to each seedling without *Pythium* inoculation. Each treatment contained six plants. Observation was terminated after 10 days. The experiment was done twice.

3.2.5. Effect of glyphosate and inoculation with *Pythium* spp. on accumulation and exudation of phytoalexins by bean roots in a hydroponic system

To study the effects of *Pythium* spp. on phytoalexin production in the hydroponic system, 1 ml of suspension of *P. ultimum* or 2 ml of *P. sylvaticum* was inoculated into each plant. After 24, 48 and 72 h, roots

were collected, blotted dry and extracted as described above. Each replicate contained three seedlings and there were three replicates within each treatment. The experiment was done twice. A second experiment involved different times of inoculation after transplanting. Seedlings were transferred to glass vials as above. Mycelial suspension was inoculated immediately or 2 or 4 days after transplanting. For the plants with delayed inoculation, the nutrient solution was replaced by fresh solution every 2 days. The roots were extracted for phytoalexins 3 days after inoculation. Each replicate contained three seedlings and there were three replicates within each treatment. The experiment was done twice.

To examine the effect of glyphosate, seedlings were grown and mycelial suspensions were prepared and inoculated as described above, either immediately after transplanting (immediate inoculation) or 2 days after transplanting (delayed inoculation). In the case of delayed inoculation, the nutrient solution was replaced by fresh sterile solution before inoculation. Glyphosate (50 μg at 5 μg μl^{-1}) was applied as described above, at the time of inoculation or immediately after transplanting in the case of treatments with delayed inoculation. All samples were collected and extracted 3 days after inoculation with *Pythium*. For treatments not inoculated with *Pythium* spp., 2 ml of 0.08% agar was added to each vial. In total, 14 treatments were applied. To extract phytoalexins in the growth medium, the bathing solution within each replicate was combined and transferred to a separatary funnel. An equal amount (v/v) of ethyl acetate was added, and the mixture was shaken for approximately 2 min. The organic phase was collected into a round-

bottomed flask. This procedure was then repeated twice. The combined ethyl acetate phase was evaporated under vacuum at 40 ^{0}C. Each treatment contained three replicates and each replicate contained three seedlings. The experiment was done three times.

3.2.6. Bioassays

Inoculated bean seeds were used to obtain the quantity of phytoalexins required for dose-response tests to assess the sensitivity of *Pythium* spp. to phytoalexins. The seeds were hydrated in tap water for 24 to 48 h. After the seed coats were removed, the seeds were chopped into small pieces and rinsed thoroughly with running tap water to remove fine debris and the soluble substances released during chopping. The chopped seeds were blotted dry. A spore suspension of *Thielaviopsis basicola* (Berk. & Broome) Ferraris prepared from 2-week old cultures grown on PDA was mixed with the chopped seeds. The mixture was air-dried for approximately 30 min and then placed in the dark at room temperature (25 ^{0}C). After 3 days of incubation, the seed pieces were collected and extracted as described above. The procedure was repeated several times to produce the required quantity of a concentrated crude extract containing 10 mg ml^{-1} phaseollin, 5 mg ml^{-1} kievitone and 3 mg ml^{-1} PIF. When injected into the HPLC, a total of ten peaks under 280 nm were detected in the crude extract. Among them, three of them were identified as the phytoalexins which composed of 67.6% of total peak area. Two other peaks of similar size, which were more soluble in water than the phytoalexins also were found. These two peaks made up 23.3% of total peak area. The remaining five peaks were very small.

Both static culture and shake culture were used to study the effect of the phytoalexin extract on mycelial growth in liquid culture. In this experiment, only bean juice liquid medium was used. For static culture, 200 µl of 95% alcohol containing different amounts of the phytoalexin extract was added to each Petri plate with 25 ml of sterile bean juice. The final concentration of phaseollin in each plate was 1, 2, 4, 8, or 16 µg ml^{-1}. The plates were inoculated and kept at 25 ^0C in the dark. For shake culture, the same amount of alcohol solution was added to flasks containing 25 ml of sterile bean juice to achieve the same range of concentrations. The same amount of alcohol solution without the phytoalexin extract was used in the controls. The flasks were inoculated and placed on a shaker at 25 ^0C and 150 rpm in the dark. After 2, 4 and 6 days of incubation, mycelia were harvested, rinsed and blotted dry. They were then dried at 80 ^0C for 24 h, and weighed after cooling. Each treatment contained two replicates.

To determine the effect of the phytoalexin extract on mycelial growth on solid media, WA and bean juice agar (BJA) (Tuite, 1969) were used. Five-hundred µl of 95% ethanol solutions containing different amounts of the phytoalexin extract was added to 25 ml of sterile distilled water, which was then added to a flask containing 25 ml of 3% WA or BJA. After mixing, 10 ml aliquots of each medium were poured into Petri plates. The final concentrations of the phaseollin component of the crude extract in individual plates were 1, 2, 4, 8 or 16 µg ml^{-1}. The same amount of 95% ethanol without the phytoalexin extract was used for the control. After the media had solidified, a 5 mm plug obtained from the margin of a *P. ultimum* or *P. sylvaticum* colony growing on CMA for 2 to 3 days was placed in the

center of each plate. The plates were kept at 25 ^{0}C in the dark. After 12, 24 and 36 h, colony diameters were measured. Two replicate plates were used for each concentration, and the experiment was done twice.

To compare the mycotoxicity of the crude extract with that of a single component phytoalexin, purified phaseollin was added to WA as described above. The final concentrations of phaseollin in individual plates were 1, 5, 10, 20 or 40 μg ml^{-1}. The same amount of 95% ethanol without phaseollin was used for the control. Due to the limited amount of phaseollin available, only the response of *P. ultimum* on WA was tested. After 12, 24 and 36 h, colony diameters were measured. Two replicate plates were used for each concentration, and the experiment was done twice.

3.2.7. Statistical analysis

The Student-Newman-Keuls test and least-squares means test were used to determine if results in different treatments were significantly different. When repeated experiments produced similar results, the data from the different experiments were combined and analyzed as a single experiment. For bioassays, a general linear model with discrete variables (medium type and time of measurement) and a continuous variable (ln concentration) was used to generate ID_{50} values for each isolate.

3.3. Results

3.3.1. Phytoalexin production by roots of bean seedlings grown in various media

Silica gel thin layer chromatography revealed nine components with absorbance at 354 nm in the extracts of roots grown in natural soil, and seven components in extracts of the portions of hypocotyls of the same seedlings in contact with soil. In both root and hypocotyl extracts, components occurred with Rf values, absorption characteristics and migration patterns on thin layers that were identical with those of authentic samples of kievitone, PIF and phaseollin. Silica gel scraped from the sample lanes in Rf regions corresponding to the phytoalexin standards and extracted with ethanol yielded UV absorption spectra with characteristics consistent with these phytoalexins (Burden *et al.,* 1972; Rahe, 1973 b; Smith *et al.,* 1973 a). Rf values of the various components of extracts are listed in Table 3.1. The authentic kievitone standard yielded a UV absorption spectrum characteristic of kievitone, with a major peak at 293 nm (Smith *et al.,* 1973 a). Direct mass spectrometry of the authentic standard yielded a parent peak with M+ = 356 which corresponded to the molecular ion of the compound. Other peaks in the spectrum were *m/e* 338, 221, 205, 192, 177, 165, and these were consistent with the published data on kievitone (Smith *et al.,* 1973 b). When the authentic kievitone standard was chromatographed on the silica gel thin layers, however, three absorbing spots were observed (Rf = 0.19, 0.23, 0.25). The component with Rf = 0.19 was the most strongly absorbing

Table 3.1. Ethyl acetate-soluble components of extracts of roots and underground hypocotyls of bean (cv. Tender Green) grown in natural soil. Silica gel thin layers were developed with benzene:ethyl acetate:formic acid (74:25:1 v:v:v) and examined under ultraviolet light (354 nm).

Rf	Root	Hypocotyl	Authentic Kievitone	Authentic Phaseollinisoflavan	Authentic Phaseollin
0.12	+	-	-	-	-
0.19	+	+	+	-	-
0.23	-	-	+	-	-
0.25	+	+	+	-	-
0.30	+	+	-	-	-
0.36	+	+	-	-	-
0.45	+	-	-	-	-
0.49	+	+	-	+	-
0.58	+	+	-	-	-
0.73	+	+	-	-	+

of these. It appeared dark brown under illumination of 354 nm and only this component yielded a UV absorption spectrum with a peak at 293 nm.

Phaseollin, PIF and kievitone were detected in the roots of seedlings of all five cultivars grown in natural soil (Figure 3.1.). Quantitative analysis showed that phaseollin and PIF occurred at 2.1 - 5.2 μg and 1.0 - 4.7 μg per g fresh weight, respectively, whereas kievitone occurred at less than 0.2 μg in all cultivars. Significantly ($P \leq 0.05$) higher concentrations of phaseollin and PIF occurred in the roots of cvs. Dark Red Kidney and Goldcrop Wax than in the other three cvs.

Phytoalexin production by roots of seedlings grown in autoclaved water agar, Metro-mix™, white silica sand, autoclaved mineral soil and natural (non-sterilized) mineral soil is illustrated in Figure 3.2. Phytoalexins were not detected in roots grown on water agar, and only trace amounts of one or more phytoalexins occurred in extracts of roots grown in sterilized soil or silica sand. The amounts of phytoalexins produced by roots of seedlings grown in autoclaved Metro-mix™ were intermediate between those produced in the other sterilized media and those produced in natural mineral soil.

Figure 3.1. Phytoalexin content in roots of five cultivars of 12-day old bean seedlings (*Phaseolus vulgaris* L.) grown in natural soil. The vertical bars indicate standard errors (n=4).

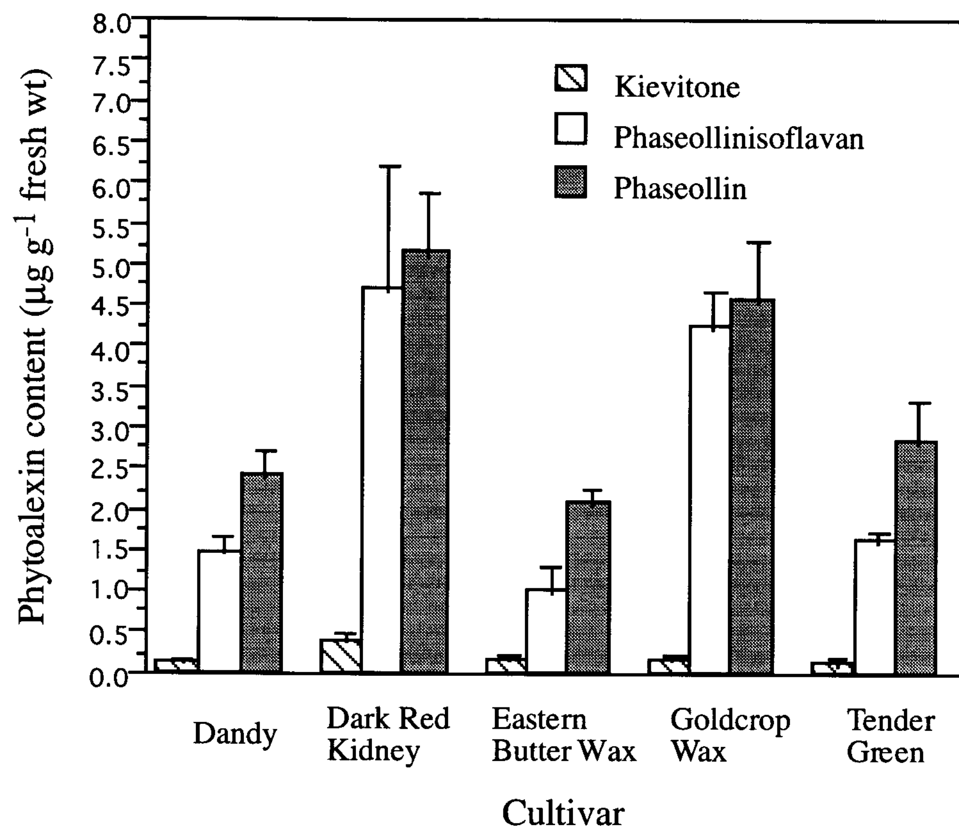
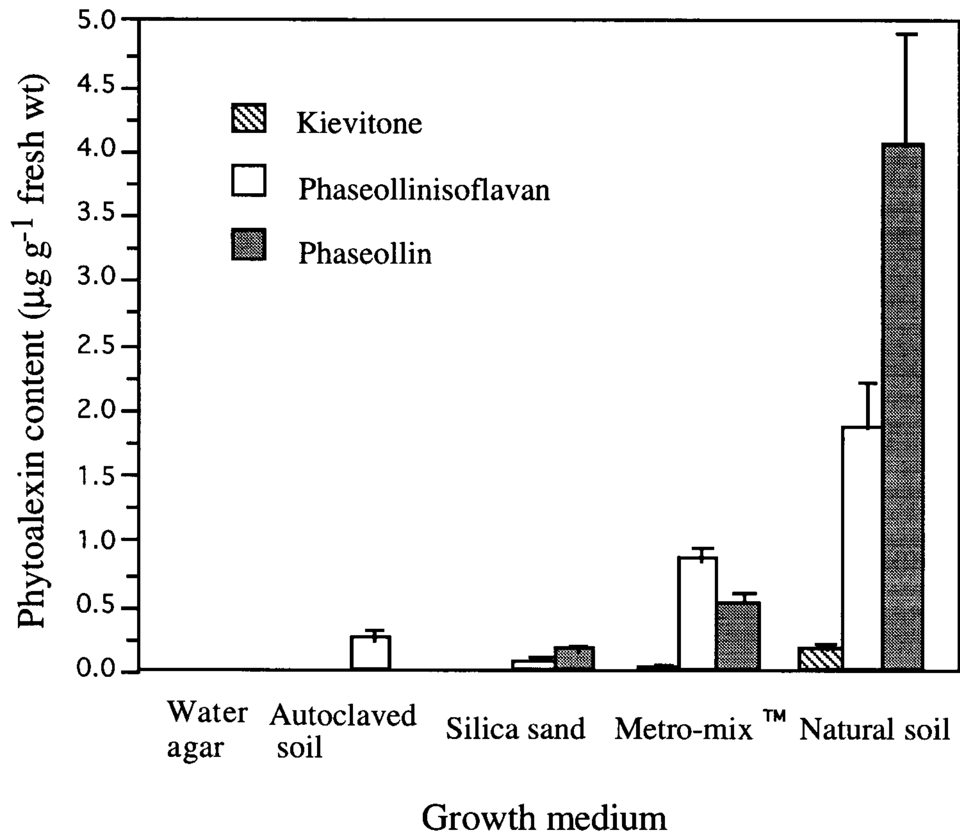

Phytoalexin content (μg g-1 fresh wt)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Kievitone
Phaseollinisoflavan
Phaseollin
Dandy
Dark Red Kidney
Eastern Butter Wax
Goldcrop Wax
Tender Green
Cultivar

Figure 3.2. Phytoalexin content in roots of 7- to 10-day old bean seedlings (cv. Tender Green) grown in different media. The vertical bars indicate standard errors (n=6).



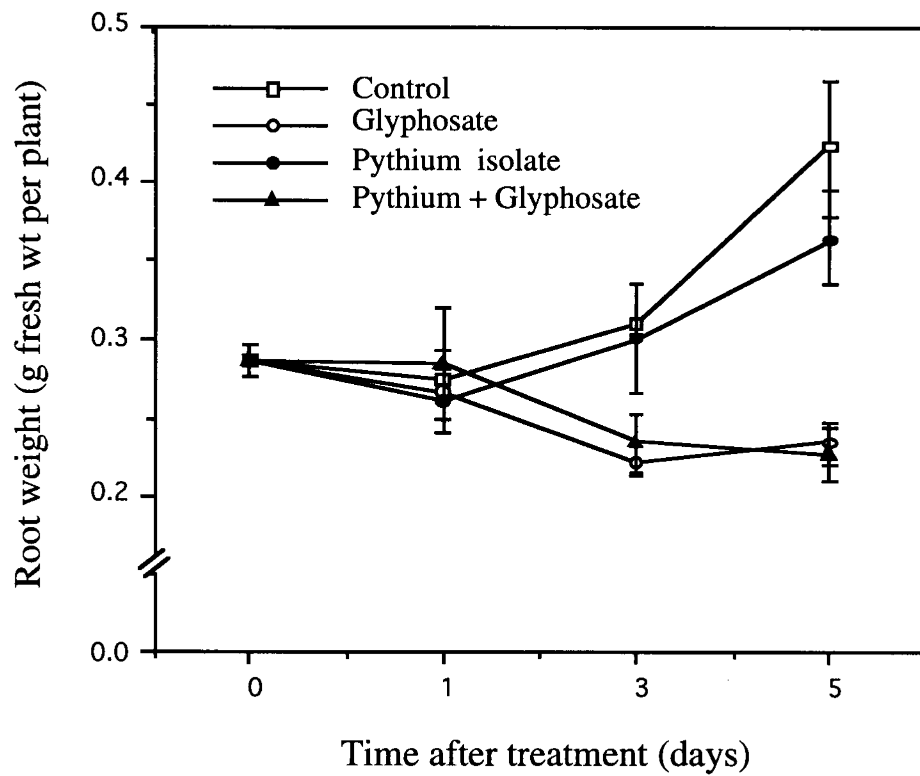
Phytoalexin content (μg g-1 fresh wt)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Kievitone
Phaseollinisoflavan
Phaseollin
Water agar
Autoclaved soil
Silica sand
Metro-mix ™
Natural soil
Growth medium

3.3.2. Effect of glyphosate and inoculation with *Pythium* spp. on phytoalexin production by roots of bean seedlings grown in silica sand

The leaves of seedlings treated with glyphosate became chlorotic within 3 days after treatment and the seedlings had shorter roots than the control plants. By 5 days after treatment, chlorosis had intensified, but no further change in the appearance of roots was observed. Root weight did not increase with time after treatment with glyphosate, whereas root weight increased with time in the control seedlings ($P \leq 0.01$). The roots of seedlings in the treatments inoculated with either *P. ultimum* or *P. sylvaticum* were darker in color than those of control plants. *Pythium* spp. alone did not cause obvious rotting of roots, whereas the roots of plants treated with both *Pythium* spp. and glyphosate had decayed by day 5. The fresh weights of roots treated with both *Pythium* spp. and glyphosate were similar to those of plants treated with glyphosate alone (Figure 3.3).

Figure 3.3. Effects of glyphosate and of inoculation with *Pythium ultimum* * or *P. sylvaticum* * on root weight of bean seedlings (cv. Tender Green) grown in silica sand. The vertical bars indicate standard errors (n=8).

* Combined data for *P. ultimum* and *P. sylvaticum* inoculation treatments; both species of *Pythium* produced similar effects.


0.5
0.4
0.3
0.2
0.0
Root weight (g fresh wt per plant)
Control
Glyphosate
Pythium isolate
Pythium + Glyphosate
0
1
3
5
Time after treatment (days)

Nil or only trace amounts (< 0.5 μg g^{-1} fresh wt) of phytoalexins were detected at any of the sampling times in the roots of bean seedlings receiving control or glyphosate treatment. In contrast, phytoalexin accumulation occurred within 3 or 5 days in all treatments that received *Pythium* inoculum (Figure 3.4, 5). Although the amounts of phytoalexins that accumulated in response to *P. ultimum* were much greater than in response to *P. sylvaticum*, the effects of both species were significant. When significant accumulation of phytoalexins occurred, concentrations of phaseollin and kievitone were higher than PIF. Glyphosate did not significantly affect the accumulation of phytoalexins at 3 days. At day 5, the levels of all three phytoalexins in treatments that received glyphosate plus *Pythium* inoculum were higher than in treatments not receiving glyphosate. The differences were significant in the case of kievitone for *P. sylvaticum* ($P \leq 0.05$) (Figure 3.4) and phaseollin for both species ($P \leq 0.01$) (Figure 3.4, 5). Since root weight of the glyphosate treated plants did not increase with time, the increased kievitone associated with glyphosate application was not significant when the data were expressed on a per plant basis, but increases of phaseollin were still significant. Levels of PIF showed an increase at day 5 with both species, but these increases were not significant (Figure 3.4, 5).

3.3.3. Determination of time of inoculation with *Pythium* spp. and time of phytoalexin collection in a hydroponic system

The seedlings showed no symptoms on either leaves or roots within 2 days for all treatments. At day 3, tissue surrounding the points where

Figure 3.4. Effect of *Pythium ultimum* and glyphosate on the accumulation of phytoalexins in roots of bean seedlings (cv. Tender Green) grown in silica sand. The vertical bars indicate standard errors (n=4). Solid dots indicate significant difference from the treatments without *Pythium ultimum* according to least-squares means test ($P \leq 0.05$). Asterisk indicates significant difference between paired bars according to least-squares means test ($P \leq 0.01$).

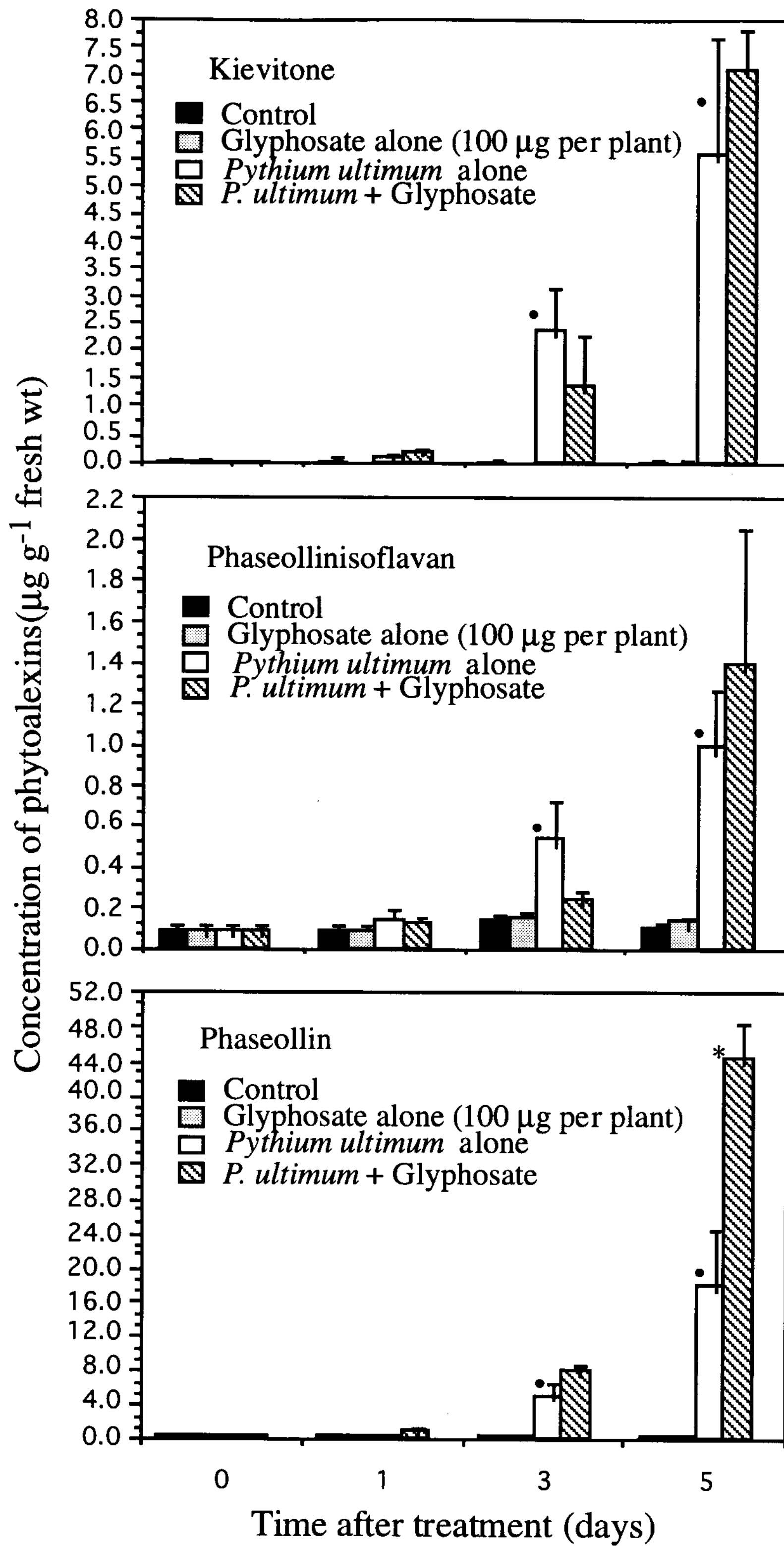
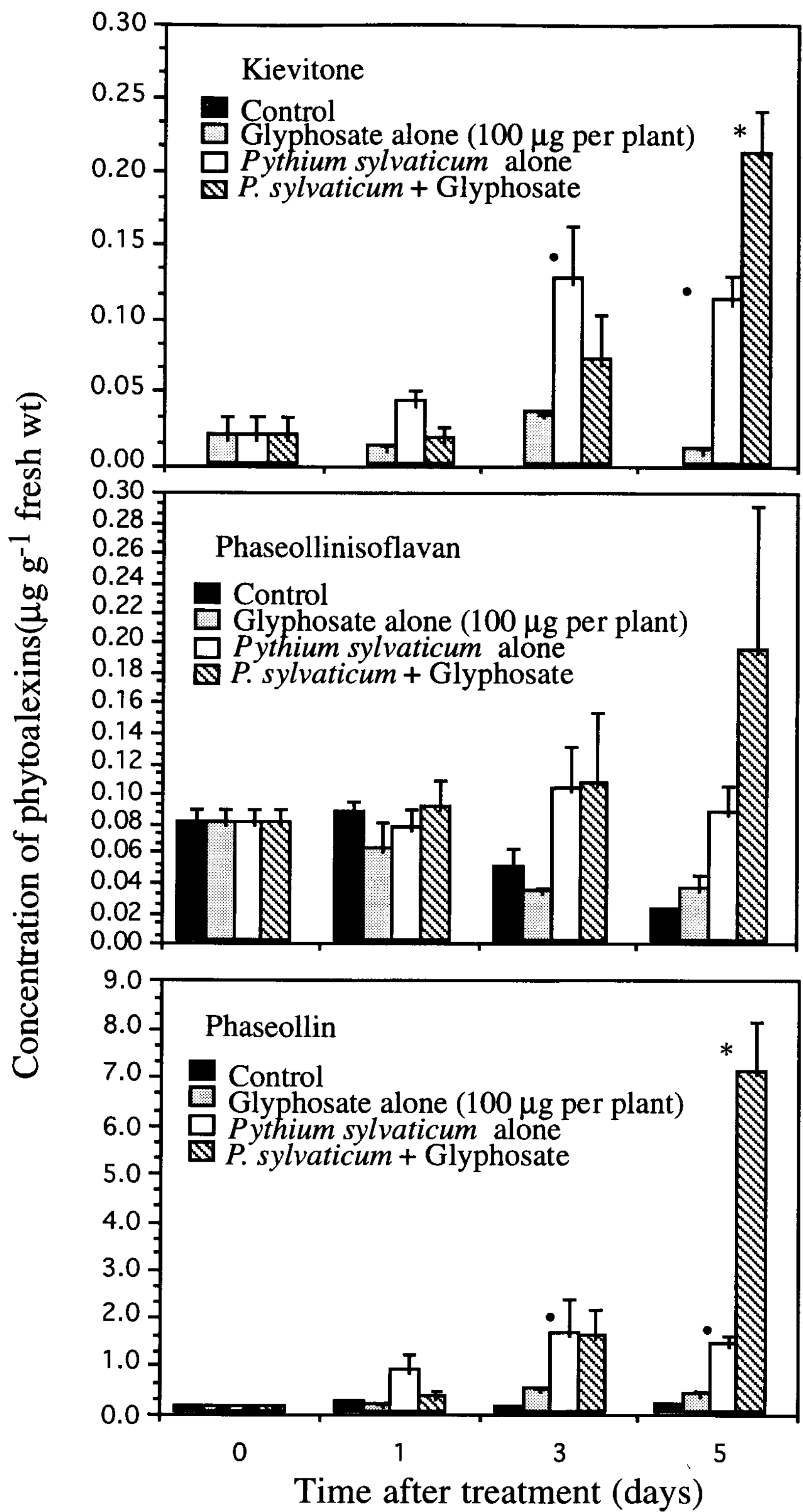

Kievitone
Control
Glyphosate alone (100 µg per plant)
Pythium ultimum alone
P. ultimum + Glyphosate
Phaseollinisoflavan
Control
Glyphosate alone (100 µg per plant)
Pythium ultimum alone
P. ultimum + Glyphosate
Phaseollin
Control
Glyphosate alone (100 µg per plant)
Pythium ultimum alone
P. ultimum + Glyphosate
Concentration of phytoalexins(µg g-1 fresh wt)
0
1
3
5
Time after treatment (days)

Figure 3.5. Effect of *Pythium sylvaticum* and glyphosate on the accumulation of phytoalexins in roots of bean seedlings (cv. Tender Green) grown in silica sand. The vertical bars indicate standard errors (n=4). Solid dots indicate significant difference from the treatments without *Pythium sylvaticum* according to least-squares means test ($P \leq 0.05$). Asterisk indicates significant difference from the treatment with *Pythium sylvaticum* alone according to least-squares means test. $P \leq 0.05$ for kievitone and $P \leq 0.01$ for phaseollin.

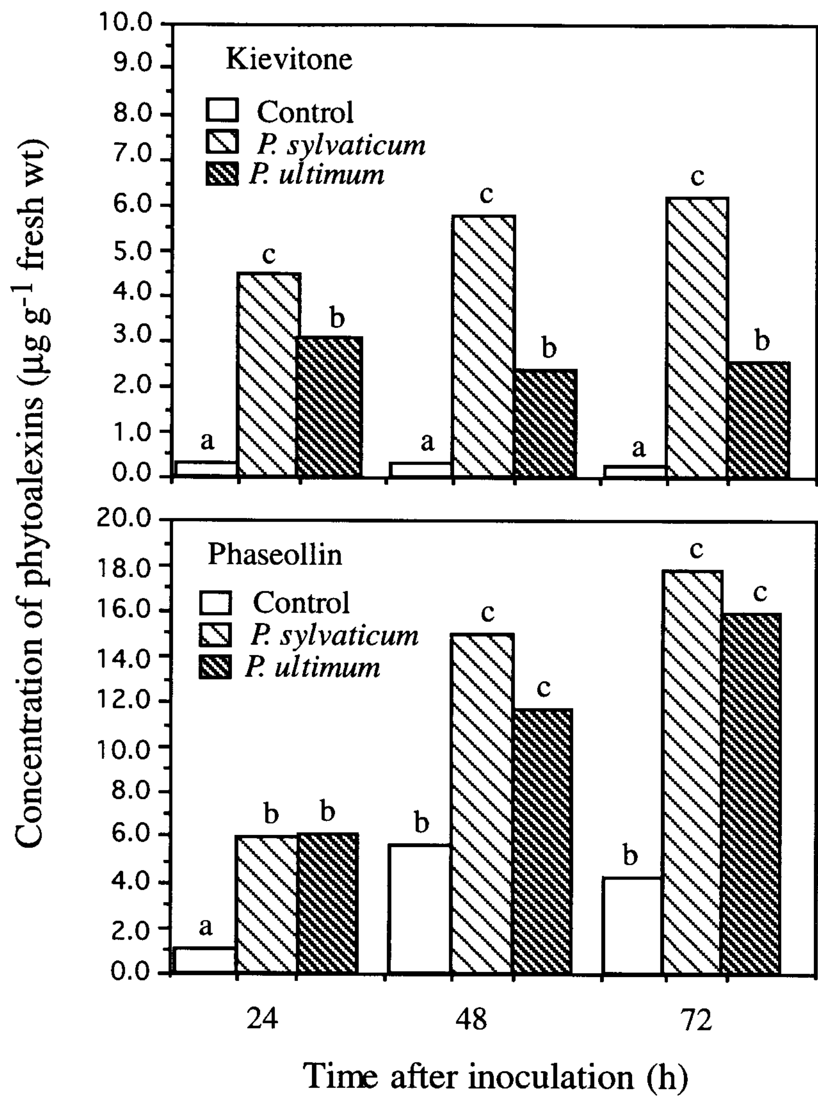

Kievitone
Control
Glyphosate alone (100 µg per plant)
Pythium sylvaticum alone
P. sylvaticum + Glyphosate
Phaseollinisoflavan
Control
Glyphosate alone (100 µg per plant)
Pythium sylvaticum alone
P. sylvaticum + Glyphosate
Phaseollin
Control
Glyphosate alone (100 µg per plant)
Pythium sylvaticum alone
P. sylvaticum + Glyphosate
Concentration of phytoalexins(µg g-1 fresh wt)
Time after treatment (days)

glyphosate was applied was chlorotic. At day 6, chlorosis on the primary leaves had intensified, but the first trifoliate leaf and the apical buds were healthy. The roots of glyphosate-treated plants without *Pythium* did not show obvious morphological change. The control plants remained healthy throughout the 10-day period of observation. At 4-6 days, new adventitious roots appeared. Adventitious roots were absent in the plants treated with glyphosate.

When treated with 25 µg glyphosate, plants inoculated with *Pythium* spp. showed no obvious symptom besides chlorosis until day 6, when some of the plants inoculated with the highest dose of either *Pythium* isolate wilted and the roots of these plants showed partial decay. The hypocotyls in the nutrient solution were colonized with mycelia. Plants treated with 50 µg glyphosate showed symptoms earlier than did plants treated with 25 µg glyphosate. Those roots started to decay at day 4. By day 8, all plants inoculated with either of the *Pythium* isolates had died or were severely wilted. All plants inoculated with the different doses of *Pythium* isolates but without glyphosate appeared as healthy as the control plants. The observations were similar for the two experiments.

Significant accumulation of kievitone and phaseollin was elicited by both *P. ultimum* and *P. sylvaticum* in the roots of bean seedlings grown in the hydroponic system at 1, 2 and 3 days post inoculation (Figure 3.6). PIF was not detected. In the control plants, the level of kievitone remained low whereas the levels of phaseollin in the control plants at 48 and 72 h were significantly higher than at 24 h. In contrast to the results

Figure 3.6. Effect of *Pythium* spp. on phytoalexin accumulation in bean roots (cv. Tender Green) in a hydroponic system. Bars with the same letter are not significantly different according to the Student-Newman-Keuls test ($P \leq 0.05$) (n=6).


Kievitone
Control
P. sylvaticum
P. ultimum
Phaseollin
Control
P. sylvaticum
P. ultimum
Concentration of phytoalexins (µg g-1 fresh wt)
Time after inoculation (h)
24
48
72
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
a
c
b
a
c
b
a
c
b
a
b
b
b
c
c
b
c
c

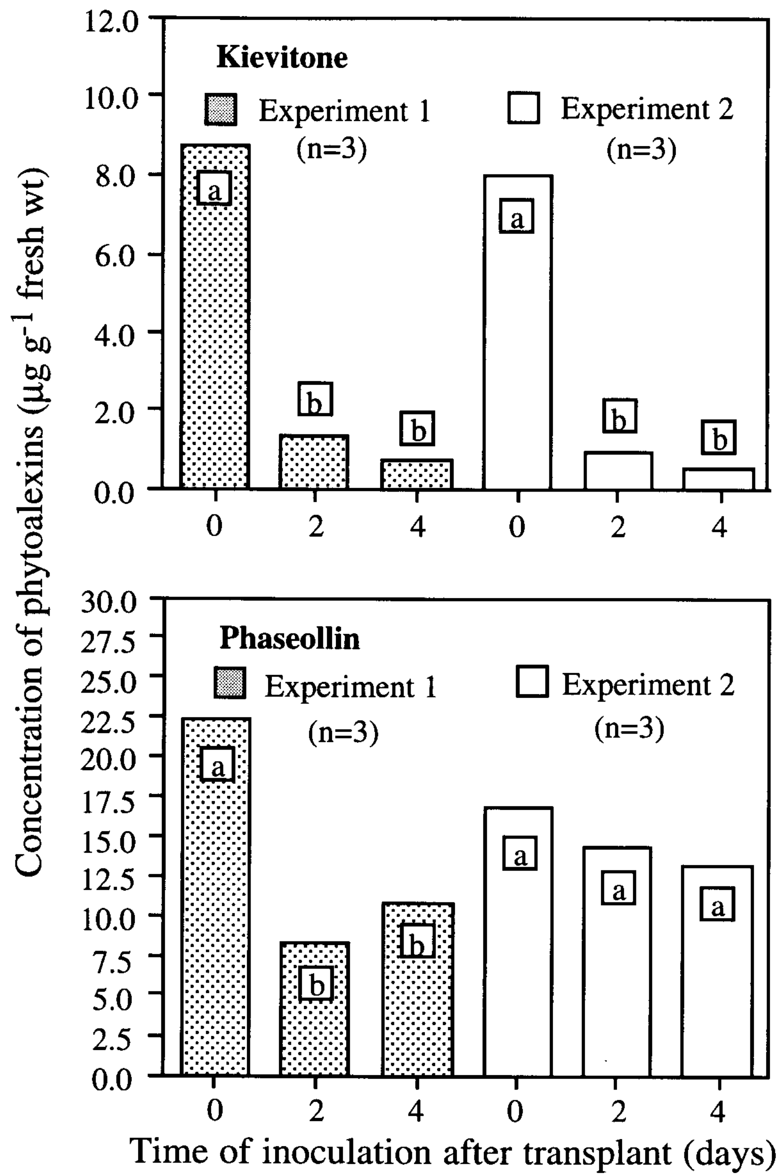
obtained in sand, *P. sylvaticum* was comparable with *P. ultimum* as an inducer in all hydroponic experiments.

Induction of kievitone was significantly less when either isolate was inoculated 2 or 4 days after transplanting than when inoculated immediately ($P \leq 0.01$), but there was no difference in induction between the 2 and 4 day inoculation treatments (Figure 3.7, 8). The results for phaseollin were inconsistent. In the first experiment, but not the second, induction was reduced for both isolates ($P \leq 0.01$) when the fungi were inoculated 2 and 4 days after transplant. As for kievitone, no difference was found in amounts of phaseollin between the 2 and 4 day inoculation treatments.

3.3.4. Effect of glyphosate and inoculation with *Pythium* spp. on accumulation and exudation of phytoalexins by bean roots in a hydroponic system

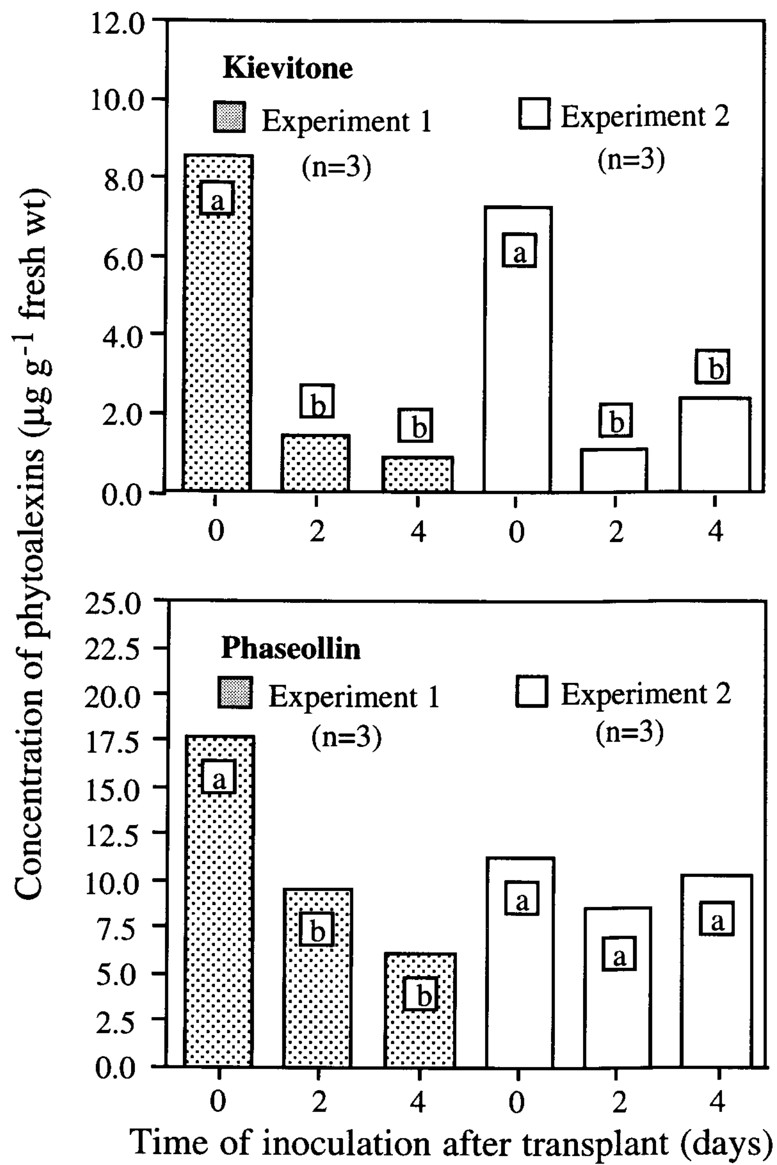
Based on the results described above, mycelial suspensions of *Pythium* spp. were inoculated at two different times (immediate or delayed relative to time of transfer from Metro-mix™ to the hydroponic system). Phytoalexins were extracted 72 h after inoculation. Accumulations of phaseollin at 72 h post inoculation were comparable for inoculations made immediately and at 2 days after transfer of seedlings into the hydroponic system. In contrast, accumulation of kievitone in the same seedlings was greater in response to immediate than to delayed inoculation ($P \leq 0.05$). Phytoalexin accumulation in roots grown in the hydroponic system was not significantly affected by glyphosate (Figure 3.9, 10).

Figure 3.7. Phytoalexin production by bean roots (cv. Tender Green) grown in a hydroponic system. *Pythium ultimum* was inoculated at different times after transplanting from Metro-mix™. Bars with the same letters within each experiment are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test.



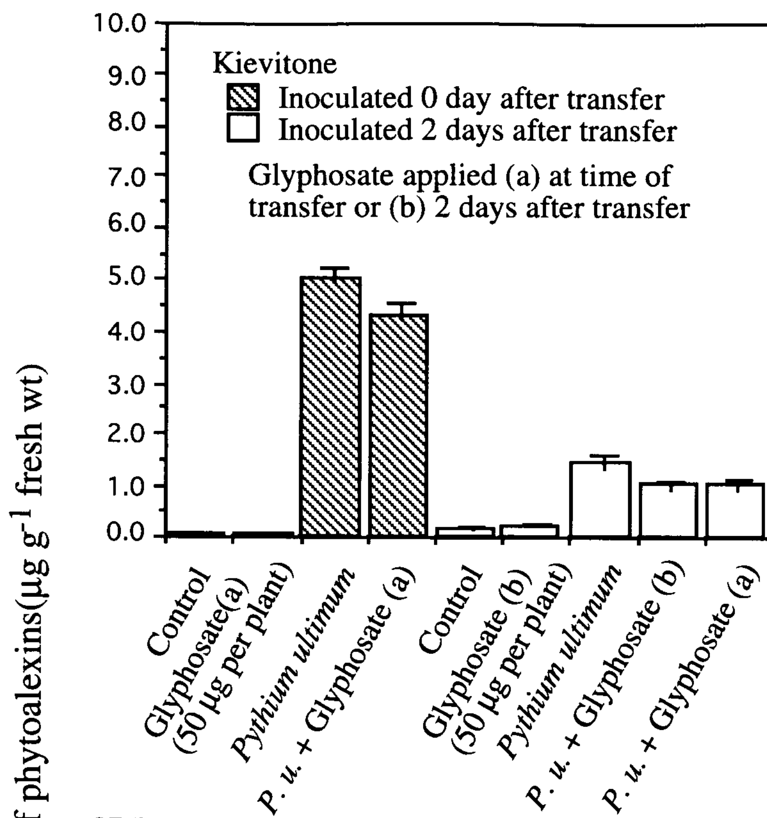
Kievitone
Experiment 1
(n=3)
Experiment 2
(n=3)
Phaseollin
Experiment 1
(n=3)
Experiment 2
(n=3)
Concentration of phytoalexins (µg g-1 fresh wt)
Time of inoculation after transplant (days)

Figure 3.8. Phytoalexin production by bean roots (cv. Tender Green) grown in a hydroponic system. *Pythium sylvaticum* was inoculated at different time after transplanting from Metro-mix™. Bars with the same letters within each experiment are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test.

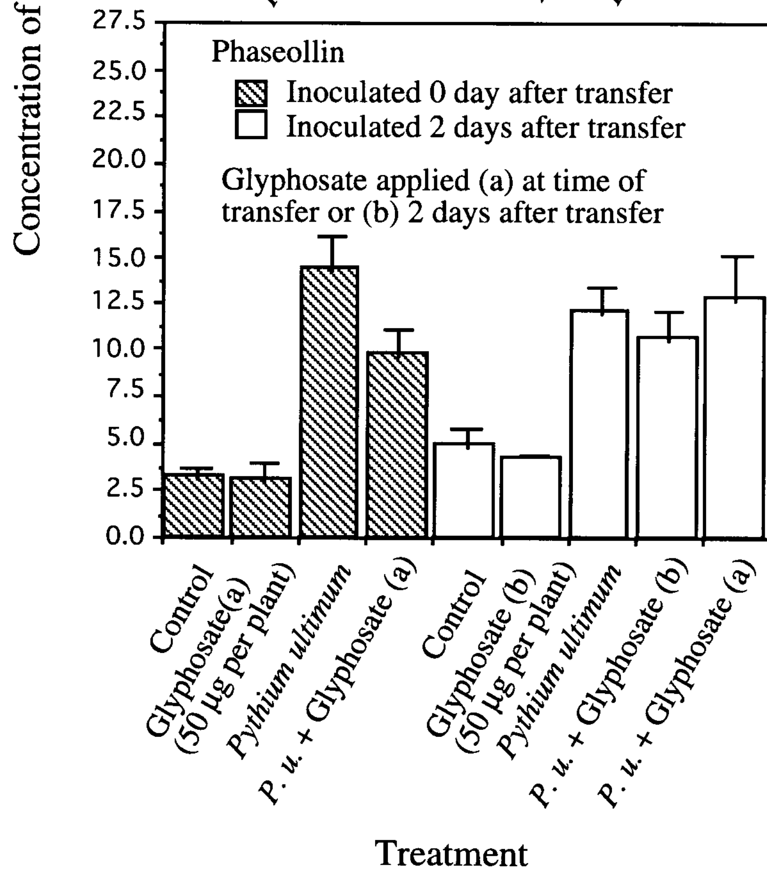


Kievitone
Experiment 1
(n=3)
Experiment 2
(n=3)
Phaseollin
Experiment 1
(n=3)
Experiment 2
(n=3)
Concentration of phytoalexins (µg g-1 fresh wt)
Time of inoculation after transplant (days)

Figure 3.9. Effect of *Pythium ultimum* and glyphosate on the accumulation of phytoalexins in roots of bean seedlings (cv. Tender Green) in a hydroponic system. Glyphosate and inoculation treatments were applied at 0 or 2 days after transfer of axenically grown seedlings into the hydroponic system, and roots were harvested at 72 h post inoculation. The vertical bars indicate standard errors (n=9).



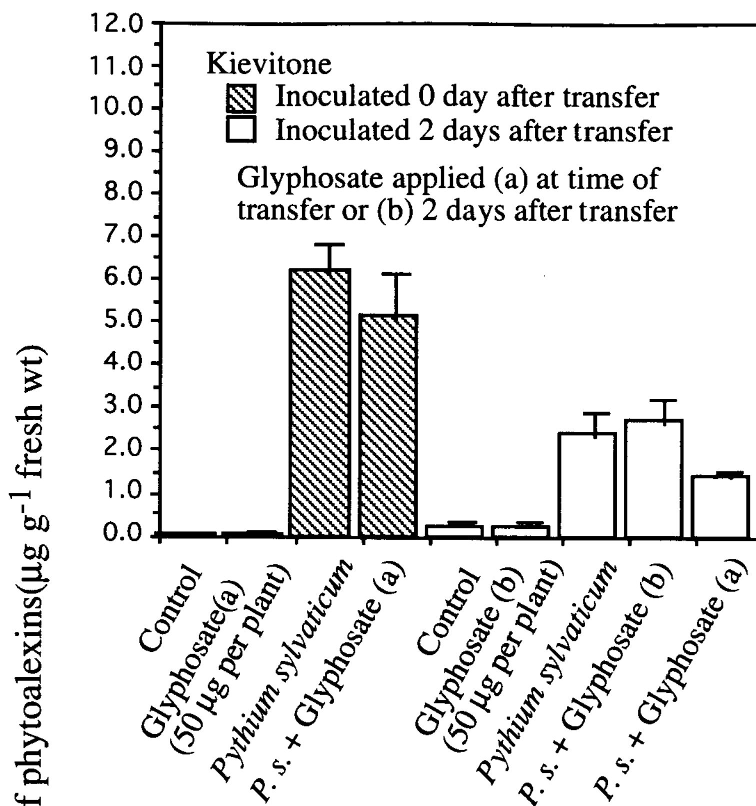
Kievitone
Inoculated 0 day after transfer
Inoculated 2 days after transfer
Glyphosate applied (a) at time of transfer or (b) 2 days after transfer
Concentration of phytoalexins(µg g-1 fresh wt)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Glyphosate(a) (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate (a)
Control
Glyphosate (b) (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate (b)
P. u. + Glyphosate (a)

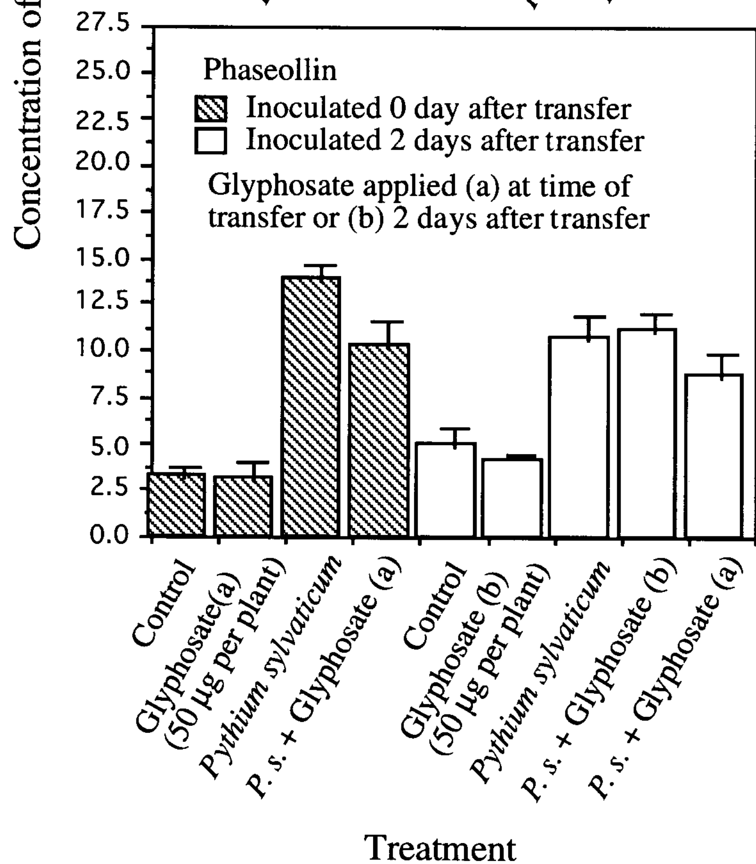


Phaseollin
Inoculated 0 day after transfer
Inoculated 2 days after transfer
Glyphosate applied (a) at time of transfer or (b) 2 days after transfer
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
Glyphosate(a) (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate (a)
Control
Glyphosate (b) (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate (b)
P. u. + Glyphosate (a)
Treatment

Figure 3.10. Effect of *Pythium sylvaticum* and glyphosate on the accumulation of phytoalexins in roots of bean seedlings (cv. Tender Green) in a hydroponic system. Glyphosate and inoculation treatments were applied at 0 or 2 days after transfer of axenically grown seedlings into the hydroponic system, and roots were harvested at 72 h post inoculation. The vertical bars indicate standard errors (n=9).

Concentration of phytoalexins(μg g^{-1} fresh wt)





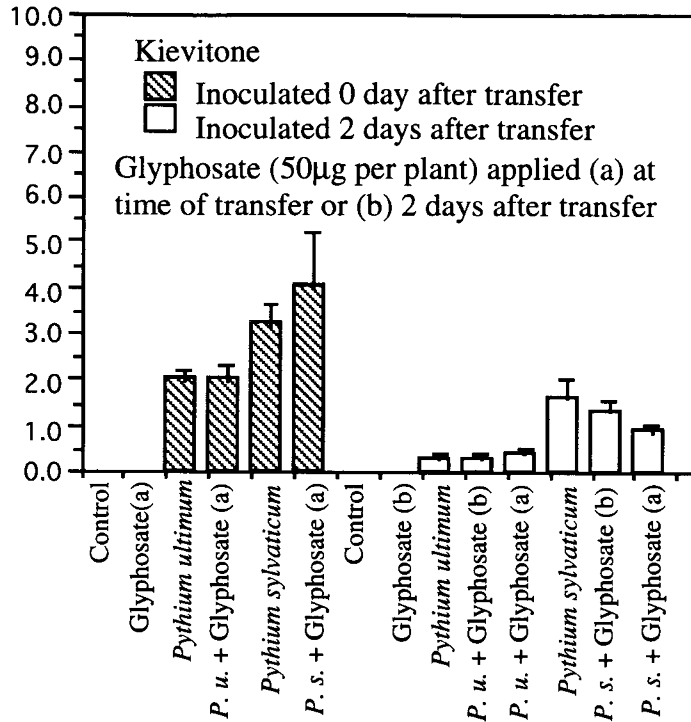
Treatment

Kievitone and phaseollin were exuded into the hydroponic solution in all treatments that involved inoculation with *Pythium* spp.; no kievitone and only small amounts of phaseollin were exuded in the non-inoculated treatments (Figure 3.11). Glyphosate treatments did not affect exudation of phytoalexins. Exudation of kievitone was greater ($P \leq 0.05$) for seedlings inoculated at the time of transfer into the hydroponic system than for seedlings inoculated 2 days after transfer, and the amounts of phaseollin exuded were similar for both times of inoculation. The amounts of phytoalexins exuded were generally proportional to the amounts of phytoalexins extracted from roots (Figure 3.11).

3.3.5. Bioassays

When *P. ultimum* was grown on WA, ID_{50} values for the bean seed extract at the three different measurement times were 3.5 to 3.6 $\mu g\ ml^{-1}$ (with respect to the concentration of phaseollin contributed by an extract containing phaseollin, kievitone and PIF in a ratio of 10:5:3). On BJA, the ID_{50} at different times varied slightly around a mean of 3.3 $\mu g\ ml^{-1}$. *P. sylvaticum* appeared to be more sensitive than *P. ultimum* on the same medium, with mean ID_{50} values of 2.5 $\mu g\ ml^{-1}$ and 2.1 $\mu g\ ml^{-1}$, respectively, on WA and BJA (Table 3.2). Coefficients of determination, R^2, ranged from 0.91-0.93 for the relationship between phytoalexin dose and mycelial growth.

Figure 3.11. Effect of *Pythium* spp. and glyphosate on exudation of phytoalexins by roots of bean seedlings grown in a hydroponic system. Glyphosate and inoculation treatments were applied at 0 or 2 days after transfer of axenically grown seedlings into the hydroponic system, and the bathing solution was extracted at 72 h post inoculation. The vertical bars indicate standard errors (n=9).


Concentration of phytoalexins in bathing solution (µg g-1 fresh wt)
Kievitone
Inoculated 0 day after transfer
Inoculated 2 days after transfer
Glyphosate (50µg per plant) applied (a) at time of transfer or (b) 2 days after transfer
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Glyphosate(a)
Pythium ultimum
P. u. + Glyphosate (a)
Pythium sylvaticum
P. s. + Glyphosate (a)
Control
Glyphosate (b)
Pythium ultimum
P. u. + Glyphosate (b)
P. u. + Glyphosate (a)
Pythium sylvaticum
P. s. + Glyphosate (b)
P. s. + Glyphosate (a)

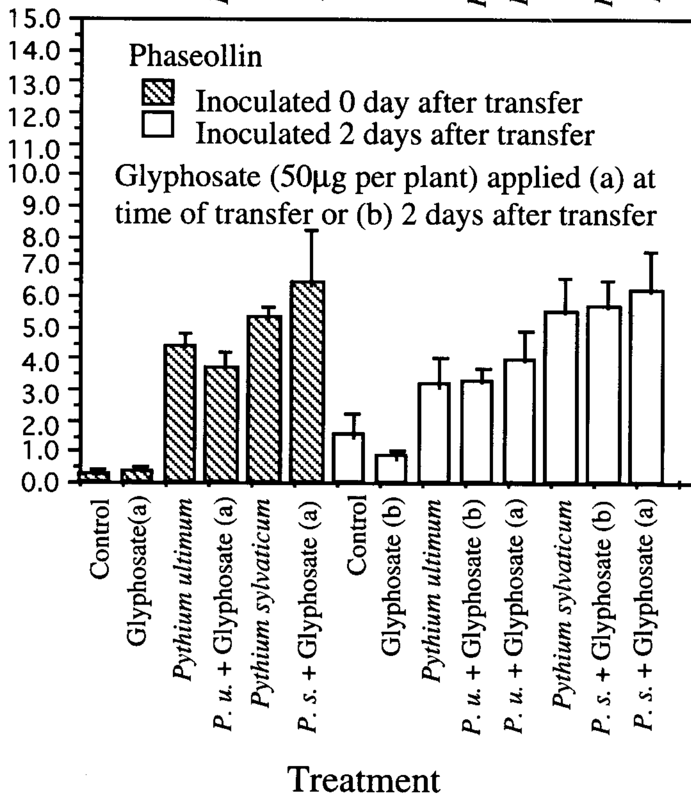

Phaseollin
Inoculated 0 day after transfer
Inoculated 2 days after transfer
Glyphosate (50µg per plant) applied (a) at time of transfer or (b) 2 days after transfer
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Glyphosate(a)
Pythium ultimum
P. u. + Glyphosate (a)
Pythium sylvaticum
P. s. + Glyphosate (a)
Control
Glyphosate (b)
Pythium ultimum
P. u. + Glyphosate (b)
P. u. + Glyphosate (a)
Pythium sylvaticum
P. s. + Glyphosate (b)
P. s. + Glyphosate (a)
Treatment

Table 3.2. ID_{50} of a phytoalexin extract obtained from inoculated bean seeds for growth of *Pythium* spp. on solid media.

Medium	Time of measurement (h)	ID50 ($\mu g\ ml^{-1}$)* *Pythium ultimum*	ID50 ($\mu g\ ml^{-1}$)* *Pythium sylvaticum*
Water agar	12	3.5	2.3
	24	3.5	2.5
	36	3.6	2.6
Bean juice agar	12	2.8	2.0
	24	3.3	2.1
	36	3.9	2.3

* Refers to the concentration of phaseollin contributed by an extract containing phaseollin, kievitone and phaseollinisoflavan in a ratio of 10:5:3.

In the plates containing purified phaseollin, ID_{50} at the different times was seven- to ten-fold higher than that of the bean seed extract: 25.6, 26.6 and 30.5 μg ml^{-1} respectively at 12, 24 and 36 h and R^2 ranged from 0.81 to 0.88.

There was little growth by either isolate at day 2 in shake culture. ID_{50} values for the bean seed extract for *P. sylvaticum* were 1.7 and 2.0 μg ml^{-1} at day 4 and day 6, respectively. The corresponding ID_{50} values for *P. ultimum* were 2.2 and 3.2 μg ml^{-1}, and R^2 in this experiment ranged from 0.76-0.90. Mycelial weights produced in static culture at different concentrations of phytoalexin were highly variable and showed no dose-response relationship.

3.4. Discussion

The roots or underground hypocotyls of growing plants are continuously exposed to microorganisms, including many which are potentially pathogenic. Under most conditions, infection does not occur and the roots remain healthy. Although broad spectrum antimicrobial substances such as phytoalexins could potentially contribute to protection of roots, information on phytoalexin production by roots is scarce in comparison with that available for various shoot tissues. Roots of bean seedlings produced phaseollin and kievitone in response to various chemical treatments, such as mercuric chloride and abscisic acid (Goossens *et al.*, 1987). In another study, uninoculated bean roots exuded phaseollin, phaseollidin, PIF and kievitone into a non-sterile liquid medium (Burden *et al.*, 1974).

I found that no or only low amounts of phytoalexins accumulated in or were exuded by the roots of bean seedlings grown axenically. In contrast, at least three phytoalexins, phaseollin, PIF and kievitone were found in the roots of bean seedlings grown in natural soil. Phaseollidin may also have been represented by a small peak that eluted between the phaseollin and PIF peaks in some of my samples, but I was unable to confirm this. My results clearly indicate that phytoalexins are produced by bean roots, and that their production is elicited by conditions, presumably microorganisms, found in natural soil. No phytoalexins were detected in roots grown on water agar, and only trace amounts occurred in the roots grown in sterilized soil and silica sand. The intermediate amounts of phytoalexins that occurred in roots of seedlings grown in sterilized Metro-mix™, and the small amounts that were associated with growth in sterilized soil and silica sand might have been due to microbial contaminants that recolonized these media during the period of seedling growth (Stössel and Magnolato, 1983), or elicited by chemicals that were released into the media by autoclaving. Alternatively, or concomitantly, minor injuries may have been caused by the coarse structure of the media and induced accumulation of low levels of phytoalexins (Rahe and Arnold, 1975). Some root injury was unavoidable during the transfer of seedlings into the hydroponic system. Nevertheless, the substantially greater accumulation associated with growth in natural soil and with inoculation with the two *Pythium* species compared with that occurring in sterile or sterilized growth media indicates that phytoalexin production by bean roots is an inducible phenomenon.

The results confirmed that glyphosate synergism, previously documented only in soil, occurred in the hydroponic system. The extent of synergism depended on the doses of glyphosate and *Pythium* inoculum in the hydroponic system. The higher the dose used, the faster the plants showed symptoms. In this system, 50 μg glyphosate did not cause obvious herbicidal injury to the plants. Since the development of symptoms, such as wilting and decay of root tissue, was slower with *P. sylvaticum* than with *P. ultimum*, a higher dose of mycelial suspension of *P. sylvaticum* than *P. ultimum* was evaluated. When combined with 1 ml of *P. ultimum* or 2 ml of *P. sylvaticum*, most of the plants were killed within 8-10 days, and these doses were used in the subsequent investigation.

The mycelial suspensions of *Pythium* spp. induced accumulation of kievitone and phaseollin by 24 h after inoculation in the hydroponic system. Accumulation of kievitone induced by both isolates did not increase with time after 24 h, but the amount of phaseollin kept increasing until 72 h. Since mycelium of *Pythium* spp. was found in treated root tissue 48 h after glyphosate application (Lévesque *et al.,* 1993 a), 72 h after inoculation was chosen for phytoalexin extraction in the further study.

Injury to the root system caused during transfer from Metro-mix™ to the hydroponic system was inevitable and uncontrollable. In the preliminary experiments, some plants were left undisturbed after transfer for 2 or 4 days before inoculation and no differences in the amounts of accumulated phytoalexins were found between these two treatments. It indicated that later inoculation provided time for the root system of the

plants to establish in the new growth environment. It is not clear why the effect of later inoculation was different for kievitone and phaseollin.

My results show that phytoalexins can be exuded from bean roots. I expected to find relatively more kievitone than phaseollin in exudates because of the greater water solubility of kievitone. This was not the case. The relative amounts of these two phytoalexins in the bathing solution mirrored their concentrations in the extracted root tissue. The concentrations of phytoalexins in the hydroponic bathing solution did not exceed 0.2 and 0.35 μg for kievitone and phaseollin on a per milliliter basis. Both *Pythium* species were insensitive to these concentrations in either the agar-based or liquid dose response tests.

In vitro tests using two species of *Pythium* gave quite different ID_{50} values for the crude phytoalexin extract and for purified phaseollin. Several possibilities could account for these differences. When purified phaseollin was added to the test medium, it may have precipitated due to its low solubility in water, thus resulting in higher ID_{50} values than would have occurred if it had been uniformly dissolved. Alternatively, kievitone, PIF or some other unknown compounds might account for most of the toxicity of the crude extract to the *Pythium* species. Synergy among compounds in the crude extract could also have enhanced its toxicity relative to that of a purified phytoalexin.

The results in this study do not support the hypothesis that glyphosate-induced predisposition of bean roots to colonization by *Pythium* spp. is due

to suppression of phytoalexin synthesis by glyphosate. Ward (1984) reported that glyphosate enhanced lesion development, but levels of glyceollin found in the glyphosate-treated tissue remained above *in vitro* ED_{90} values. Glyceollin appeared to be much less active *in vivo* than *in vitro*, or it did not accumulate uniformly in the lesions. If *Pythium* was the elicitor for phytoalexins in the bean root tissues of my study, the phytoalexins must have accumulated in tissues not occupied by *Pythium* spp.. It is conceivable that *Pythium* elicited phytoalexin accumulation without colonizing the root tissue. However, glyphosate-enhanced colonization of roots by *Pythium* occurred within 3 days in this study and in other comparable studies (Lévesque *et al.,* 1993 a; Descalzo *et al.,* 1995). An alternative possibility is that while glyphosate removes the barrier to colonization present in untreated plants, *Pythium* elicits phytoalexin accumulation as an adjacent cell response by penetrating cells. *Pythium* itself could escape the toxic effects of the elicited phytoalexins by rapid growth, keeping ahead of the area of phytoalexin accumulation. This hypothesis is consistent with the fact that the fast-growing *P. ultimum* was a more effective elicitor than the slow growing *P. sylvaticum* in silica sand, where the roots were presumably attacked by mycelia growing from the inoculated agar discs.

When mycelial homogenates were used to inoculate a hydroponic medium, the two fungal species were of comparable efficacy. Elicitation by fungal metabolites or leachates from the macerated mycelium could have occurred in the hydroponic system, in which case differences in growth rates of the two fungi would not have been a factor. If the ID_{50} of phaseollin to *Pythium* spp. provided by using the purified phaseollin is

considered reliable, the infection of *Pythium* spp. in bean roots could be explained by tolerance of the fungi to the phytoalexins produced in root tissue since the ID_{50} value exceeded the concentration accumulated in root tissue inoculated with *Pythium* spp.. The phytoalexin accumulation may not be important in resistance of bean roots to colonization by *Pythium* spp. under these circumstances

Johal and Rahe (1988, 1990) reported that suppression of phytoalexin production in bean hypocotyls inoculated with an incompatible race of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Scribner. depended on conditions that competed for or depleted phenylalanine reserves, such as exposure to light and removal of cotyledons. The results presented in this research demonstrated that glyphosate induced symptoms characteristic of this herbicide but failed to suppress the production of phytoalexins in the root system, even though it is a demonstrated inhibitor of phenylalanine synthesis, a major precursor of isoflavonoid phytoalexins. To further stress the plants by removing cotyledons or/and increasing the dose of glyphosate applied may change the outcome. Based on the results obtained in this study, I conclude that predisposition by glyphosate of bean roots to colonization is not due to suppression of phytoalexin accumulation or exudation.

Chapter IV

Effect of *Pythium* spp. and Application of Glyphosate on lignification in Bean Roots

4.1. Introduction

Lignin is an important constituent of the cell walls of all vascular plants. Aside from cellulose, it is the most abundant polymer in plants and is commonly found in the walls of plant cells that have a supporting or mechanical function. It is a polymeric natural product arising from enzyme-initiated and chemically driven dehydrogenative polymerization of primary precursors possessing a *p*-hydroxycinnamyl alcohol structure (Sarkanen and Ludwig, 1971).

In addition to its structural role, lignin and the lignification process of plants have been implicated as important factors in the resistance response of plants against potential pathogens (Vance *et al.*, 1980; Nicholson and Hammerschmidt, 1992). Ride (1978) postulated that lignin or the lignification process could act in plant defense against infection by establishing mechanical barriers to pathogen growth. Lignified cell walls could be more resistant to cell-wall degrading enzymes and toxins released by pathogens, and could reduce the amount of nutrients exuded from the host tissues to the pathogen.

Non-pathogenic fungi induced rapid lignin synthesis in plant tissues and fungal growth was limited to infection sites (Ride, 1975; Hammerschmidt, 1984). Hyphae of pathogenic fungi failed to penetrate lignified papillae formed opposite to the penetration points (Ride and Pearce, 1979; Bird and Ride, 1981; Edwards and Ayres, 1981). The contents of lignin were found to be inversely correlated with rates of infection by pathogens (Evans and Stephens, 1989; Brown and Lee, 1993). The resistant cultivars of plant species produced lignin rapidly and extensively upon infection by pathogens (Werner and Siwecki, 1978; Henderson and Friend, 1979; Southerton and Deverall, 1990; Reimers and Leach, 1991). Activities of the enzymes that are involved in lignification were enhanced in resistant plants after pathogen infection. Phenylalanine ammonia lyase (PAL) activity in roots of resistant *Eucalyptus* species increased within 24 h of inoculation with *P. cinnamomi* (Cahill and McComb, 1992). In pearl millet seedlings resistant to downy mildew (*Sclerospora graminicola* (Sacc.) J. Schröt), the activity of PAL increased in shoot tissue. In contrast, PAL activity decreased in shoot, mesocotyl and root of susceptible plants (Nagarathna *et al.,* 1993). When tomato near-isogenic lines were inoculated with the nematode *Meloidogyne incognita*, activity of peroxidase in resistant isolines doubled (Zacheo *et al.,* 1993).

A few reports have shown a relationship between high lignin content and resistance in the root systems of plants. Most of these deal with woody plants. *P. cinnamomi* elicited lignin formation in roots of *Eucalyptus calophylla* L. (field resistant), whereas lignin concentrations in inoculated roots of *E. marginata* Donn ex Sm (field susceptible) were unchanged

(Cahill and McComb, 1992). When stands of Douglas-fir were inoculated with *Armillaria ostoyae* after different thinning and fertilization treatments, root bark of the trees with the least infection by *A. ostoyae* had the highest lignin concentration (Entry *et al.,* 1991). Resistance of tomato roots to Fusarium crown and root rot was associated with the incorporation of phenolic or lignin-like materials and suberin within cell walls (Brammall and Higgins, 1988 a). Cahill *et al.* (1989) reported that when zoospores of *P. cinnamomi* penetrated the roots of a group of plant species ranging from fully susceptible to fully resistant, lesions on roots of the resistant plants were localized. Lignification of cell walls, deposition of phenolics and the formation of callosic papillae were more commonly observed in the resistant species than in the susceptible ones.

Applying inhibitors of lignification may change the resistance response of plants. Treatment of roots of *E. calophylla* with the PAL inhibitor, aminooxyacetate, altered the resistance response through changes in the concentration of lignin and phenolics (Cahill and McComb, 1992). The same inhibitor also reduced the deposition of lignin as a response to non-pathogenic fungi in potato tuber and induced a susceptible reaction in the tissue (Hammerschmidt, 1984). Tiburzy and Reisener (1990) reported that aminooxyacetate inhibited the hypersensitive reaction of wheat seedlings to *Puccinia graminis* f. sp. *tritici* Erics. & E. Henn, and markedly altered resistance expression. Other research on resistance of wheat to stem rust showed that treatments with several competitive inhibitors of PAL decreased the frequency of lignified necrotic cells of wheat leaves and concomitantly led to increased fungal growth (Moerschbacher *et al.,* 1990).

When applied to plants as sublethal doses, glyphosate shifts host-pathogen interactions toward increased disease (Johal and Rahe, 1984; Keen *et al.,* 1982; Bramall and Higgins, 1988 b). Increased fungal colonization was associated with inefficient incorporation of phenolic materials into the cell walls (Bramall and Higgins, 1988 b). Glyphosate, an inhibitor of aromatic amino acid synthesis, has been reported to reduce lignification in various plant tissues (Sharma, 1986; Saltveit, 1988). The objective of this chapter was to determine the effect of glyphosate on deposition of lignin in root systems grown in media with or without glyphosate - synergistic *Pythium* spp. using bean as a model plant.

4.2. Materials and Methods

4.2.1. Effect of glyphosate on lignin content of roots of bean seedlings grown in Metro-mix™

Bean seeds (cv. Tender Green) were surface sterilized with 1.2 % NaOCl for 5 min, then rinsed several times with sterile distilled water. The surface sterilized seeds were planted individually into autoclaved Metro-mix™ in Styrofoam coffee cups. The cups were kept at 25 ^{0}C with 14 h photoperiod and light intensity of 240 µmol m^{-2} s^{-1}. The seedlings were watered with distilled water when necessary. After 14 days, Roundup® diluted with distilled water to 10 µg glyphosate μl^{-1} was applied to the upper surface of the primary leaves of the seedlings. Each leaf received five droplets. Totally, 100 µg of glyphosate was applied to each

plant. The same amount of sterile distilled water was applied to control plants.

The root tissues of seedlings were collected 8, 24, 72 and 120 h after application of glyphosate, and the fresh weight of the roots was measured. Each treatment contained fibrous roots from five seedlings. The content of lignin was determined by the thioglycolic acid procedure (Barber and Ride, 1988). The roots from each treatment were put together and extracted in four changes of methanol over a period of 48 h. After the extracted roots were dried at room temperature (25 ^{0}C), 10 mg tissue taken from each dried sample were ground in 5 ml of 0.5 M NaOH, transferred to 15 ml graduated centrifuge tubes individually, and incubated at 25 ^{0}C for 24 h to hydrolyze cell wall bound phenolic acids. A total of three-10 mg replicates were taken from each sample. The mixture in each tube was neutralized with 1 ml of 2.0 M HCl and the residues were collected by centrifugation. Each residue was then washed with 10 ml of water, collected by centrifugation, and resuspended in 10 ml of methanol and collected by centrifugation (3000 x g for 10 min). The air-dried solids were treated with 10 ml of 2.0 M HCl and 1 ml of thioglycolic acid and incubated in sealed tubes at 95 ^{0}C for 4 h. After centrifugation, the solids in each tube were washed twice by resuspension in 5 ml of water followed by centrifugation. The supernatant was discarded. To solubilize the ligninthioglycolic acid (LTGA), the solids were incubated with 10 ml of 0.5 M NaOH for 16 h. After incubation, insoluble material was removed by centrifugation and washed twice by resuspension in 2 ml of water, followed by centrifugation. The sodium hydroxide extract and the water

washes were combined in a 50-ml graduated centrifuge tube and 2 ml of concentrated HCl was added. The solution was held at 4 C for 48 h to aid LTGA precipitation. The precipitated LTGA was collected by centrifugation, resuspended in 2 ml of 0.1 M HCl and held at 4 C for 48 h. The resuspension in 2 ml of 0.1 M HCl step was repeated after centrifugation. The precipitated LTGA was collected by centrifugation and dissolved in 3 ml of 0.5 M NaOH at 25 C for 24 h. Insoluble material was removed by centrifugation (12,000 x g for 3 min). The final extracts were diluted five-fold in 0.5 M NaOH and the absorbance was measured at 280 nm. The experiment was done twice.

4.2.2. Effect of inoculation with *Pythium* spp. and application of glyphosate on the lignin content of roots of bean seedlings grown in a hydroponic system

Seedlings were grown in hydroponic culture (Chapter III). Mycelial suspensions of *Pythium* spp. were inoculated at the same doses either immediately after transplanting (immediate inoculation) or 2 days after transplanting (delayed inoculation). Glyphosate treatments (50 μg at 5 μg μl^{-1}) were applied at the time of inoculation or immediately after transplanting in the case of treatments with delayed inoculation (Chapter III). The roots were collected and extracted 3 days after inoculation with *Pythium* spp.. The tissues were weighed and processed as described above except for the final precipitated LTGA which was dissolved in 2 ml of 0.5 M NaOH. These final extracts were diluted as described above. The

absorbance was measured at 280 nm. There were three replicates in each treatment. The experiment was done twice.

4.2.3. Statistical analysis

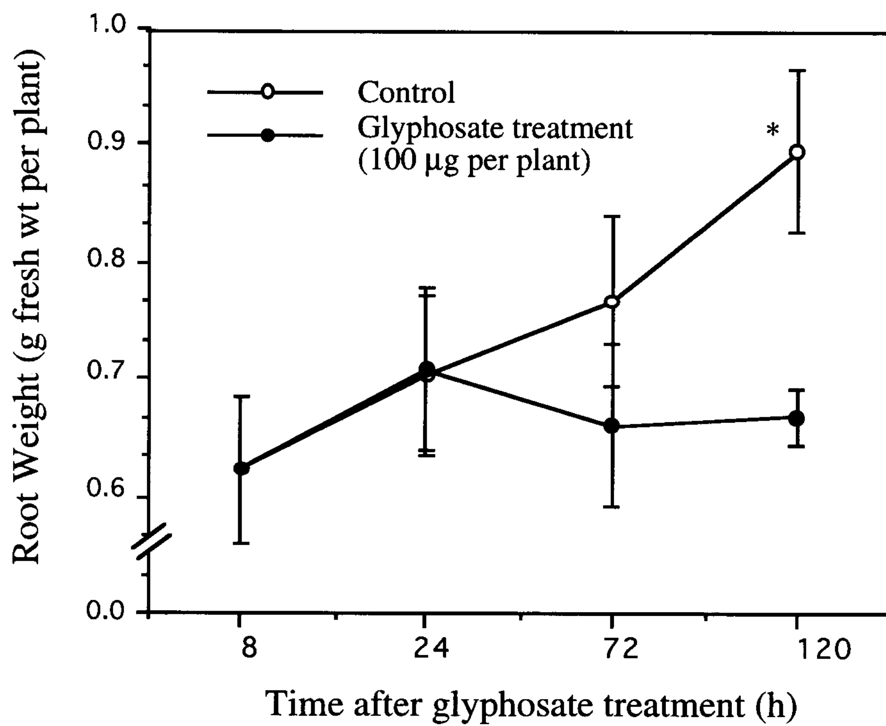
Data were analyzed by least-squares means tests. When repeated experiments produced similar effects, the replicates among different experiment were combined.

4.3. Results

4.3.1. Effect of glyphosate on the lignin content of roots of bean seedlings grown in Metro-mix™

Root weight did not increase with time after treatment of the primary leaves of bean seedlings with glyphosate, whereas root weight increased with time in the control seedlings ($P \leq 0.01$) (Figure 4.1). Alkali-soluble, acid-insoluble LTGA was obtained by thioglycolic acid procedure. The UV-absorbance spectra produced by LTGA in 0.5 M NaOH from the non-treated and the treated plants were similar. Maximum absorbance was observed at 280 nm and was consistent with published information (Southerton and Deverall, 1990; Whitmore 1978). When a final NaOH extract was diluted, the absorbance at each dilution correlated positively with the concentration (R^2=0.91) The data present here were the actual absorbance of the diluted samples representing 2 mg dried root tissue. For

Figure 4.1. Effect of glyphosate on root weight of bean seedlings (cv. Tender Green) grown in Metro-mix™. The vertical bars indicate standard errors (n=6). Asterisk indicates significant difference between control and experimental treatment according to least-squares means test ($P \leq 0.01$).

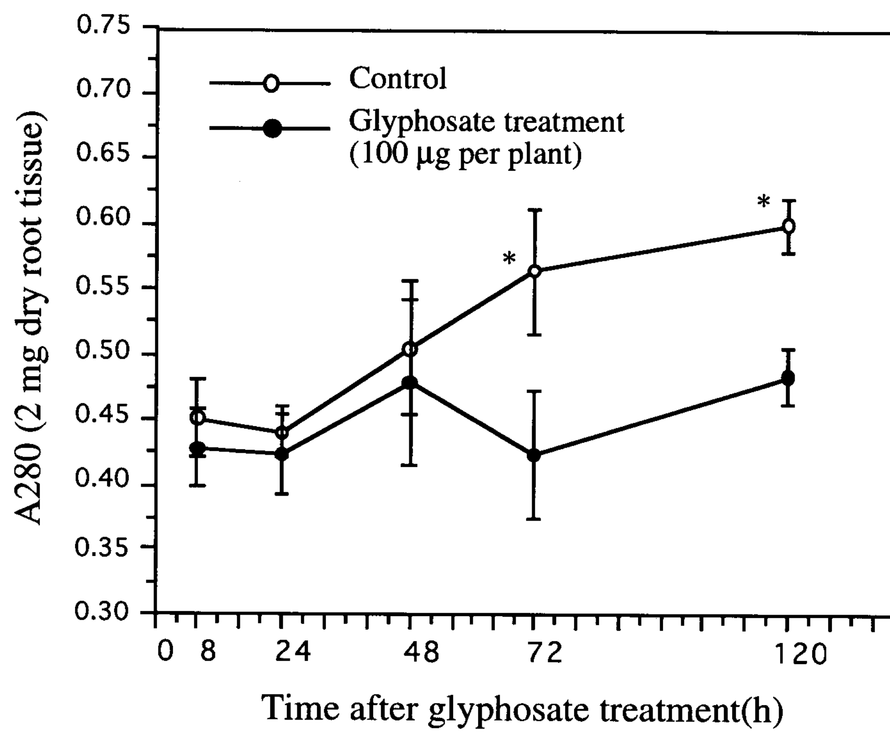

Root Weight (g fresh wt per plant)
1.0
0.9
0.8
0.7
0.6
0.0
Control
Glyphosate treatment
(100 µg per plant)
*
8
24
72
120
Time after glyphosate treatment (h)

the samples from the control plants, absorption at 280 nm increased with time. At 72 and 120 h, absorbances were significantly higher than at 8 and 24 h ($P \leq 0.05$). In contrast, no significant differences were found among the absorbances of the samples from treated plants during the period of observation. When compared with the treated plants, absorbance of the control was significantly higher than that of the treated plants at 72 and 120 h ($P \leq 0.05$) (Figure 4.2).

4.3.2. Effect of *Pythium* spp. and application of glyphosate on the lignin content of roots of bean seedlings grown in a hydroponic system

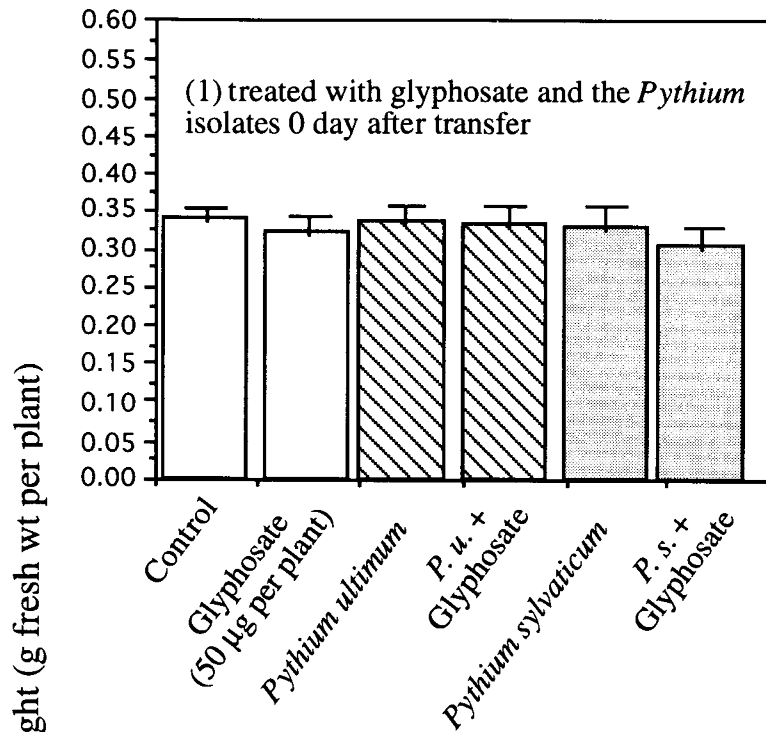
Root weights of the plants from all treatments in the hydroponic system were similar (Figure 4.3). In this system, glyphosate alone did not affect lignin deposition (Figure 4.4, 5). When seedlings were treated immediately after transfer into the hydroponic system, the lignin content of the roots did not differ significantly among the treatments (Figure 4.4). When seedlings were treated 2 days after transfer, concentrations of lignin from the seedlings inoculated with the *Pythium* isolates were higher than those in control ($P \leq 0.05$). Lignin contents decreased when glyphosate was applied simultaneously with inoculation of *Pythium* spp., but significantly only in the treatments where glyphosate was applied 2 days prior to inoculation ($P \leq 0.05$) (Figure 4.5).

Figure 4.2. Effect of glyphosate on the lignin content in the roots of bean seedlings grown in Metro-mix™. Lignin content was measured as absorbance of ligninthioglycolic acid under 280 nm. The vertical bars indicate standard errors (n=6). Asterisks indicate significant difference between control and treatment according to least-squares means test ($P \leq 0.05$).



Control
Glyphosate treatment
(100 µg per plant)
A280 (2 mg dry root tissue)
Time after glyphosate treatment(h)
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0
8
24
48
72
120
*
*

Figure 4.3. Effect of *Pythium* spp. and glyphosate on the fresh weight of roots of bean seedlings grown in hydroponic culture. The root weight was measured 72 h after inoculation. Glyphosate was applied (a) at the time of inoculation with the *Pythium* isolates or (b) 2 days prior to inoculation of the *Pythium* isolates. The vertical bars indicate standard errors (n=6). No significant difference between treatments was found according to least-squares means test (P>0.05).


Root Weight (g fresh wt per plant)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
(1) treated with glyphosate and the Pythium isolates 0 day after transfer
Control
Glyphosate (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate
Pythium sylvaticum
P. s. + Glyphosate

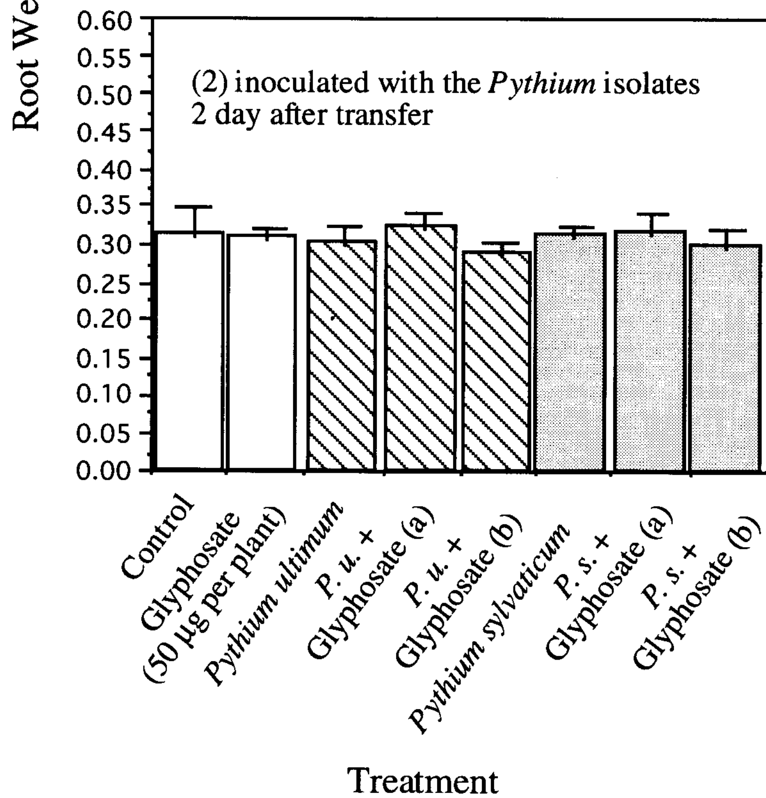

0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
(2) inoculated with the Pythium isolates 2 day after transfer
Control
Glyphosate (50 µg per plant)
Pythium ultimum
P. u. + Glyphosate (a)
P. u. + Glyphosate (b)
Pythium sylvaticum
P. s. + Glyphosate (a)
P. s. + Glyphosate (b)
Treatment

Figure 4.4. Effect of glyphosate and inoculation of *Pythium* spp. on the lignin content of roots of bean seedlings grown in a hydroponic system. The treatments were applied immediately after transferring the seedling into the hydroponic system. Glyphosate was applied simultaneously with inoculation of *Pythium* spp. Lignin content was measured as absorbance of ligninthioglycolic acid under 280 nm. The vertical bars indicate standard errors (n=6). No significant difference between treatments was found according to least-squares means test ($P>0.05$).

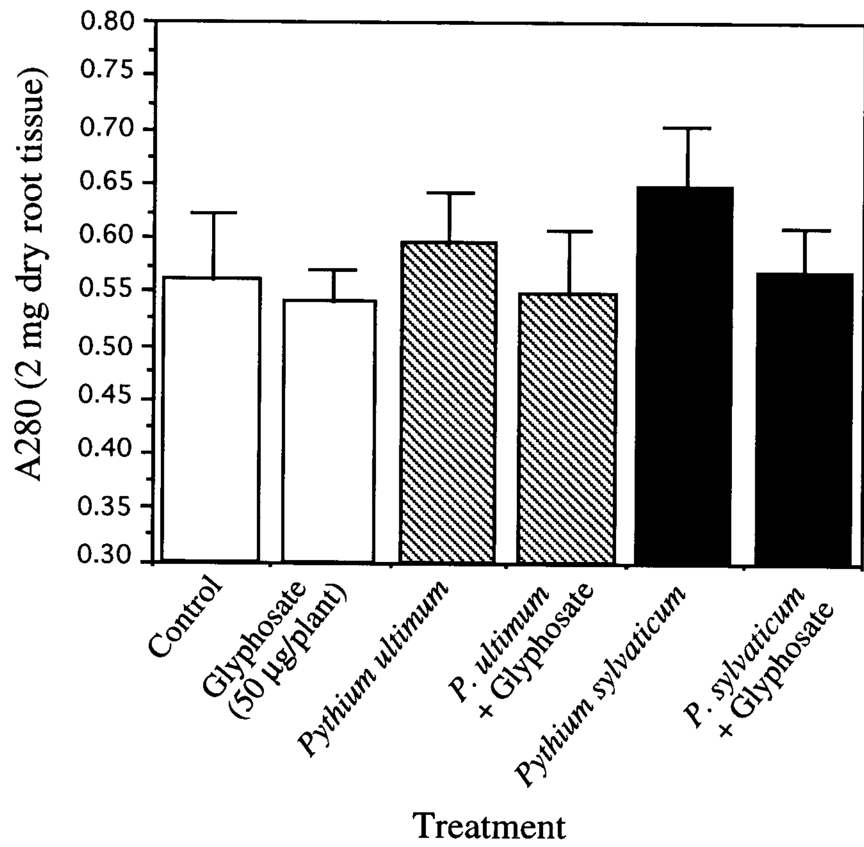
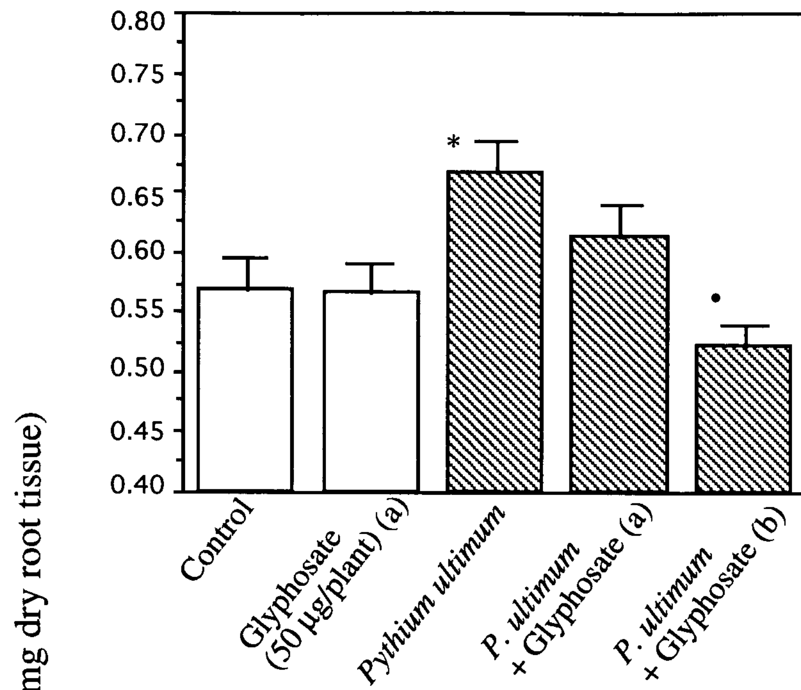
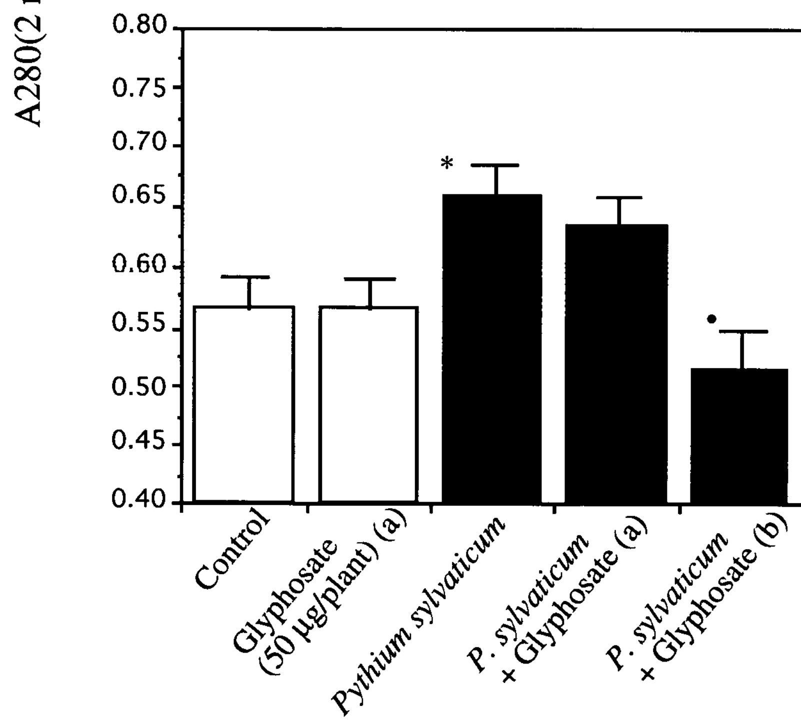

0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
A280 (2 mg dry root tissue)
Control
Glyphosate (50 µg/plant)
Pythium ultimum
P. ultimum + Glyphosate
Pythium sylvaticum
P. sylvaticum + Glyphosate
Treatment

Figure 4.5. Effects of glyphosate and inoculation of *Pythium* spp. on the lignin content of roots of bean seedlings grown in a hydroponic system. The seedlings were inoculated with *Pythium* spp. 2 days after transferring the seedlings into the hydroponic system. (a) glyphosate was applied 2 days after transfer. (b) glyphosate was applied immediately after transfer. Lignin content was measured as absorbance of ligninthioglycolic acid under 280 nm. The vertical bars indicate standard errors (n=6). Asterisks indicate significant difference from the treatments without *Pythium* spp. and solid dots indicate significant difference from the treatments with *Pythium* inoculation alone according to least-squares means test ($P \leq 0.05$).


A280(2 mg dry root tissue)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
*
•
Control
Glyphosate (50 µg/plant) (a)
Pythium ultimum
P. ultimum + Glyphosate (a)
P. ultimum + Glyphosate (b)


0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
*
•
Control
Glyphosate (50 µg/plant) (a)
Pythium sylvaticum
P. sylvaticum + Glyphosate (a)
P. sylvaticum + Glyphosate (b)
Treatment

4.4. *Discussion*

Lignin is normally found in the xylem, sclerenchyma and bundle sheath cells in higher plants. In Metro-mix™, where *Pythium* spp. were not involved, the data represent structural lignin content in the roots of bean seedlings. The lignin content of roots increased as the control seedlings grew older in the medium. Pretreatment with 0.5 M NaOH to hydrolyze cell wall-bound phenolic acids did not decrease the yields of LTGA, indicating non-interference by wall-bound phenolic compounds. A similar increase was not observed in the plants treated with glyphosate. Lignification can be regulated by the supply of lignin precursors, during transport of lignin monomers into the cell wall, and during polymerization of hydroxycinnamyl alcohols to form the lignin material in the cell wall (Grisebach, 1981). The results of my research are consistent with the hypothesis that glyphosate may inhibit lignin synthesis in plants by reducing the supply of lignin precursors derived from phenylalanine from the shikimic acid pathway.

In the hydroponic system, glyphosate alone did not appear to stop lignin deposition in the treated plants as it did in Metro-mix™. This apparent lack of effect of glyphosate on lignification in the hydroponic system may be because root growth, and presumably lignification, of the roots of the control seedlings grown in the hydroponic system was not as fast as that in the solid media. The fact that root weight in the control plants did not increase with time in the hydroponic system as it did in the solid media such as Metro-mix™ indicates that the roots of the control

plants were not growing actively. When application of glyphosate and inoculation with mycelial suspensions of *Pythium* spp. were carried out immediately after transfer, the lignin contents in the different treatments varied, but no significant differences were found. The root systems of seedlings were unavoidably injured when the seedlings were lifted from Metro-mix™ and transferred to the nutrient solution. Phenylalanine ammonia-lyase and cinnamic acid-4-hydroxylase, two key enzymes in the phenylpropanoid pathway, were found to accumulate in wounded hypocotyl tissue of French bean (Smith *et al.,* 1994). Various plant tissues in different plant species stained positive for lignin after wounding (Rittinger *et al.,* 1987). The effects of *Pythium* spp. and glyphosate may have been confounded by the physiological changes caused by injury to the roots. Delaying the treatments to provide a period of time for wound healing was used to minimize this confounding effect.

In the delayed inoculations, both *Pythium* isolates were found to stimulate lignification in roots. Application of glyphosate 2 days prior to *Pythium* inoculation inhibited the *Pythium* - induced lignification. The magnitudes of induced lignification and suppression of lignification by glyphosate were moderate. Previous research showed that glyphosate-induced colonization of bean roots by *Pythium* involved an average of only two to three colony forming units (CFU) per root system at 48 h after glyphosate treatment (Lévesque *et al.,* 1993 a). Southerton and Deverall (1990) found that the lignin contents in leaves of wheat were related to the numbers of cells that had undergone hypersensitive reaction and to dose of inoculation. Histochemical work confirmed that lignification was

associated with epidermal cells around sites of penetration (Ride, 1975; Werner and Siwecki, 1978). If the numbers of infection on bean roots occurring in the hydroponic system used in my research were low, the moderate changes in lignification associated with the inoculation of *Pythium* spp. and the application of glyphosate could reflect large changes at the sites of infection.

The number of infection sites was not estimated in my study, but the roots of inoculated seedlings without glyphosate appeared healthy within 72 h. It is possible that lignification at infection sites was abolished by application of glyphosate, thereby eliminating a barrier to spread of *Pythium* in the root system. Considering that glyphosate-enhanced infection occurred at a very low frequency, reduction of lignification at localized infection sites by glyphosate may have been a significant factor in enhancement colonization of roots of glyphosate-treated plants by *Pythium spp.*.

Chapter V

Conclusions and Future Work

Since the commercial formulations of glyphosate are widely used in agriculture and urban environments, understanding of the mechanisms of glyphosate predisposition of roots of plants to fungal invasion is of practical as well as academic significance. Based on available information on interactions of herbicides and diseases of plants, this thesis was focused on the effects of glyphosate on radial growth and sporangial germination of *Pythium* spp., root exudation, phytoalexin production and exudation by roots, and effects on lignification in root systems using bean as a model plant.

5.1. The possible role of exuded glyphosate by roots on mycelial growth and sporangial germination of Pythium spp. in soil

Based on the recommended rates for application of Roundup® for most weed problems (8 - 25 μg of glyphosate per cm^2) (Rahe *et al.,* 1990), a range of concentrations of glyphosate was chosen to study the direct effect of glyphosate on *Pythium* spp. The results demonstrated that the chosen concentrations of glyphosate did not stimulate mycelial growth of the tested fungi significantly. However, 1 μg ml^{-1} glyphosate enhanced germination of sporangia of *P. ultimum* significantly. Radioactively labelled glyphosate was found in root exudates 24 h after [^{14}C]glyphosate was applied to foliage of *Agropyron repens* (Coupland and Caseley, 1979).

Sporangia of *P. ultimum* usually germinate rapidly in response to stimulants present in seed or root exudates (Nelson, 1990). Germ tubes produced by sporangia were probably the primary means of infection by *P. ultimum* (Agnihotri and Vaartaja, 1967). As root exudates from glyphosate treated plants can contain unmetabolized glyphosate, there is the possibility of effects on soil microorganisms in the rhizosphere even though it is generally considered that exuded glyphosate will be adsorbed on to soil constituents and biodegraded (Sprankle *et al.,* 1975 a, b).

5.2. Effect of glyphosate on colonization of bean roots by Pythium spp. mediated through root exudation

Glyphosate is known to be translocated from foliage to roots readily after application (Sandberg *et al.,* 1980). Brecke and Duke (1980) reported that integrity of bean leaf cells (cv. Eastern Butterwax) was not affected after glyphosate treatment. Whether glyphosate can change the permeability of roots has not bean studied. My results show that the root exudates from glyphosate-treated plants had a stimulatory effect on germination and growth of sporangia as early as 6 h after foliar application. It is likely that glyphosate can change the integrity of the cell membranes of root cells, resulting in exudates that could favor sporangial germination of *Pythium* spp.. The changes in root exudation of glyphosate-treated plants may have been an important factor in glyphosate predisposition.

5.3. Changes in defense mechanisms of host plants after glyphosate treatment

GSF elicited accumulation of phytoalexins in bean roots (Chapter III). However, the results in this thesis demonstrated that enhanced fungal colonization of root systems was not due to a reduction of phytoalexin accumulation by glyphosate in roots of treated plants inoculated with GSF. Application of glyphosate has been shown to reduce lignin content in treated tissues (Sharma, 1986; Saltveit, 1988). The effect of glyphosate increased with time between application and detection and with concentration (Saltveit, 1988). My results showed that lignin content of bean roots grown in Metro-mix™ without inoculation of *Pythium* spp. was significantly reduced after 72 h. In the hydroponic system, the content of lignin was reduced only when glyphosate was applied 2 days prior to inoculation of GSF. Apparently, reduction of lignin content by glyphosate was not rapid enough to explain the glyphosate predisposition. However, previous results have showed that an average of only 2-3 CFU per bean root were found in bean roots grown in natural soil after glyphosate treatment (Lévesque *et al.,* 1993 a). In my research, 1 or 2 ml of mycelial suspension (10^4 CFU ml^{-1}) was added to each seedling grown in 20 ml of nutrient solution. Exact CFU in the root system after glyphosate treatment was not studied. However, the inoculated root of glyphosate-treated plants did not show any sign of decay within 3 days. Those roots started to decay in some of the seedlings at day 4. By day 7, roots in most treated plants were severely decayed. The progress of the symptom development was similar to that described in previous research (Lévesque *et al.,* 1993 a).

Considering that glyphosate-enhanced infection occurred at a very low frequency, reduction of lignification at localized infection sites by glyphosate may have been significant to enhance the colonization by *Pythium*.

5.4. Future Work

This research demonstrated that root exudates collected at 6 and 12 h after glyphosate treatment stimulated sporangial germination of *P. ultimum*. Effect of glyphosate on root exudation may have played a very important role in glyphosate synergism. Attention should be given to the spectrum of amino acids in root exudates due to the mode of action of glyphosate. However, effect of glyphosate on amino acids in the root exudates collected at 6 and 12 h after glyphosate treatment was not studied. Nelson and Craft (1989) reported that sporangia of *P. ultimum* collected from synthetic media germinated rapidly in response to sugars and amino acids. However, sporangia produced in association with plant tissue failed to respond to the same compounds. Other molecules in root exudates rather than amino acids may be involved in enhancement of sporangial germination. The exact nature of the stimulatory molecules in the exudates should be investigated.

This research was focused on (1) direct effects of glyphosate on spore germination and on mycelial growth of *Pythium* spp. and indirect effects on fungal propagules and growth mediated through root exudates; (2) effects that altered the resistance of bean plants to soil fungi. Effects of

sublethal doses of glyphosate on populations of *Pythium* spp. and on antagonists of *Pythium* spp. in soil can be another objective to study.

My results demonstrated that glyphosate reduced synthesis of lignin but had no significant effect on accumulation of phytoalexins in bean roots. The shikimic acid pathway is essential for synthesis of both lignin and isoflavonoid phytoalexins in higher plants. The reduction of the preexisting pool size of phenylalanine by glyphosate can not explain the different effects of the herbicide on phytoalexins and lignin. Two molecular forms of EPSP synthase were found in *Euglena gracilis* L. and the two enzyme forms were localized in different subcellular compartments and inversely regulated at the protein and mRNA levels during light-induced chloroplast development (Reinbothe, *et al.* 1994). The question whether a glyphosate-insensitive EPSP isozyme exists in plants for synthesis of phytoalexins needs to be investigated.

It appears that the accumulation of phytoalexins is not very important in resistance of bean roots to *Pythium* spp.. To understand defense mechanisms in root system, a pathogen causing localized necroses in roots can be used with glyphosate to study effects of glyphosate on lignin content in cells of host plants close to infection sites.

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