THE NATURE AND SIGNIFICANCE OF FUNGAL COLONIZERS IN THE HERBICIDAL EFFECT OF GLYPHOSATE

by

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ABSTRACT

Seedlings of three monocotyledonous and two dicotyledonous plant species were less sensitive to glyphosate when grown in heat treated soil than in raw soil. *Pythium* spp. and *Fusarium* spp. were not detected in heat treated mineral or organic soils at the time of glyphosate treatment, although fungi of several other genera were present. The efficacy of glyphosate on wheat or beans grown in heat treated mineral soil was recovered when untreated aqueous soil extracts were added to the heat treated soil. Colony forming units of fungal colonizers per root system were estimated. Fungal colonization of roots of wheat and bean seedlings grown at a 25:18°C day:night regime took place less that 48 h after glyphosate treatment. *Pythium* spp. were the most frequent colonizers of glyphosate treated plants and *Fusarium* spp. were the second most frequent colonizers. Under each of four imposed environmental conditions (a combination of two temperatures, 17°C and 25°C, and two soil matric potentials, -0.06 and -1.0 bar), *Pythium* spp. were the predominant colonizers. There was a reduction in *Pythium* colonization at 17°C compared to 25°C but moisture had no significant effect. *Fusarium* spp. were the second most frequent colonizers and their colonization was higher in the dry (-1.0 bar) condition. Sixty-one different *Pythium* isolates from glyphosate treated wheat or bean seedlings grown in mineral or organic soils were examined with regard to possible host and site (soil type) specificity. Almost all isolates were identified as *P. sylvaticum*, found more predominantly in roots of seedlings grown in mineral soil, and *P. ultimum*, found more predominantly in roots of seedlings grown in organic soil. Differences in repeated DNA sequences detected by

restriction endonuclease digestion and electrophoresis as well as hybridization with cloned and labelled DNA probes were used for differentiating the isolates within species. DNA variation within species did not correlate with soil type or host from which the isolates were obtained. Two isolates from each fungal species were selected for *in vivo* pathogenicity and host specificity experiment. All isolates reduced seedling emergence in wheat but only *P. ultimum* caused a reduction of emergence in beans. There was no indication of host specialization among the isolates tested, in the sense of greater pathogenicity toward seeds of a plant species by *Pythium* isolates obtained from that plant species compared with those obtained from the other plant species.

RÉSUMÉ

Les plantules de trois espèces de plantes appartenant aux monocotylédones et de deux espèces appartenant aux dicotylédones étaient moins susceptibles au glyphosate lorsqu'elles poussaient dans un sol traité à la chaleur que lorsqu'elles poussaient dans un sol non traité. Les champignons appartenant aux genres *Pythium* et *Fusarium* ne purent être décelés dans les sols de type minéral ou organique traités à la chaleur bien que des champignons de plusieurs autres genres étaient présents. L'efficacité du glyphosate sur le blé ou les fèves plantés dans un sol minéral traité à la chaleur fut rétablie par l'addition à ce sol traité d'un extrait aqueux provenant du même type de sol mais non traité. L'infestation des racines par les champignons fut estimée de façon quantitative. L'infestation des plantules de blé et de fèves qui poussaient sous un régime jour-nuit de 25-18°C eut lieu moins de 48 h après l'application du glyphosate. Sous les quatre conditions environnementales imposées (une combinaison de deux températures, 17°C et 25°C, et deux niveaux d'humidité du sol, -0.06 et -1.0 bar), les champignons du genre *Pythium* étaient le plus fréquemment isolés des racines. L'infestation des racines par ces champignons était moins prononcée à une température de 17°C comparativement à 25°C mais les différents niveaux d'humidité du sol imposés n'eurent pas d'effet significatif. Les champignons du genre *Fusarium* étaient les deuxièmes en importance et leur taux d'infestation était le plus élevé lorsque le sol était sec (-1.0 bar). Soixante et un isolats différents de champignons du genre *Pythium* furent isolés des racines de plantules de blé et de fèves traitées au glyphosate poussant dans des sols de type organique et minéral et furent examinés pour

déterminer leur spécificité au niveau de la plante hôte et du type de sol. Presque tous les isolats appartenaient aux espèces *P. sylvaticum*, retrouvé plus fréquemment dans les racines de plantules poussant dans le sol minéral, et *P. ultimum*, retrouvé plus fréquemment dans les racines de plantules poussant dans le sol organique. Les différences de séquences répétitives d'ADN visibles après digestion par endonucléases de restriction et électrophorèse ainsi que l'hybridation de fragments d'ADN clonés et marqués furent utilisées pour différencier les isolats de même espèce. Les variations au niveau de l'ADN à l'intérieur de chaque espèce ne correspondèrent pas avec l'hôte et le type de sol d'où provenaient les isolats. Deux isolats de chaque espèce de champignon furent sélectionnés pour un test *in vivo* de pathogénicité et de spécificité au niveau de l'hôte. Tous les isolats réduisirent la germination du blé mais seulement *P. ultimum* réduisit la germination des fèves. Il n'y eut aucun indice de spécialisation des isolats de *Pythium* envers leur hôte dans le sens d'une plus grande pathogénicité envers les graines de l'espèce de plante sur laquelle un isolat avait originalement été trouvé qu'envers l'autre espèce de plante.

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INTRODUCTION

An immense diversity of microorganisms use plant tissues as substratum for growth. Healthy plants harbor a very diverse microflora in their rhizospheres and on leaves but are normally protected from internal colonization by natural defense mechanisms. Plant aging, stress and other factors can interfere with natural defenses and transform plant tissue into a substratum that can be exploited by a succession of microorganisms (Bruehl 1975). Microorganisms that are the first to occupy the plant substratum have a great advantage over others and further improve their competitive edge by utilizing the unexploited nutritive elements of the tissue (Bruehl 1987). While primary colonization in plant disease and succession of colonizers in natural decay have been extensively studied, relatively little attention has been directed to general colonization of plants following treatment with herbicides.

Herbicides and plant diseases

Most research papers about the interaction between herbicides and plant diseases deal with direct effects of herbicides on growth of plant pathogenic fungi, or with the incidence of a given disease after application of herbicides. Katan and Eshel (1973), Altman and Campbell (1977), Griffiths (1981), Smith (1982), and Altman and Rovira (1989) have reviewed this literature. Herbicides do not always shift plant-pathogen interactions toward increased disease. For example, Bruckart *et al.* (1988) demonstrated that low levels of bentazon applied to improve the efficacy of the rust fungus, *Puccinia canaliculata* (Schw.) Lagerh., for biological control of yellow nutsedge (*Cyperus esculentus* L.),

instead reduced disease level by 50%. Resistance of tomatoes (*Lycopersicon esculentum* Mill.) to vascular wilt caused by *Fusarium* spp. or *Verticillium* spp. was increased by various herbicides (Grinstein *et al.* 1984; Gentile and Bovio 1986). It should not be taken for granted that herbicides coming in contact with plants necessarily increase susceptibility to diseases or make the plants available to saprophytes.

Glyphosate

Glyphosate is a broad spectrum, water soluble herbicide sold under the trade names of Kleenup, Roundup, Rodeo and Vision. It can control effectively 76 of the world's worst 78 weeds (Franz 1985). In 1981, the sales of Roundup® reached $410 million and sales of over $1 billion were projected for 1986 (Grossbard and Atkinson 1985). Because of the insolubility of glyphosate in organic solvents and of the presence of multiple polar functional groups, analysis of plant tissues or soil to estimate glyphosate concentration is challenging. The official method of analysis is gas chromatography but research efforts to develop techniques for routine detection are ongoing (Tuinstra and Kienhuis 1988; Roy and Konar 1989; Thompson *et al.* 1989). Despite these technical difficulties, there is a growing body of literature describing the fate of this herbicide in the agroecosystem.

Glyphosate is readily translocated throughout the plant within a few days after treatment (Sprankle *et al.* 1975c; Sandberg *et al.* 1980) and can be recovered intact in the roots or as its major metabolite, aminomethylphosphonic acid (Marshall *et al.* 1987). As plants progress toward flowering or dormancy,

their ability to translocate glyphosate to the roots decreases (Harvey *et al.* 1985; Willis *et al.* 1989).

Glyphosate is readily adsorbed to soil particles through ion exchange and hydrogen bonding (Sprankle *et al.* 1975a, 1975b; Glass 1987; Miles and Moye 1988; Roy *et al.* 1989) or forms insoluble metal complexes (Subramaniam and Hoggard 1988). It can be degraded by various microorganisms (Balthazor and Hallas 1986; Pipke *et al.* 1987; Pipke and Amrhein 1988; Quinn *et al.* 1988; Heinonen-Tanski 1989) or by ultra violet light (Lund-Høie and Firestad 1986). There was no evidence of effects on litter decomposition, insects or water fauna after application of the herbicide (Fletcher and Freedman 1985; Holck and Meek 1987; Kreutweiser *et al.* 1989) but a decrease in population of aquatic bacteria was recorded after application of the herbicide (Chan and Leung 1986). Glyphosate does not seem to be acutely toxic if ingested (Moses 1989) but the surface active agent polyoxyethyleneamine present in the formulation can be lethal if ingested (Sawada *et al.* 1988). The effects of glyphosate on the environment were reviewed by Carlisle and Trevors (1988).

Mode of action of glyphosate

There is strong evidence that the primary target of glyphosate is an enzyme of the shikimic acid pathway: 5-enolpyruvylshikimate-3-phosphate synthase (Jaworski 1972; Steinrüken and Armhein 1980, 1984; Cole 1985). Because of the absence of the shikimic acid pathway in mammals and because of all the environmental considerations described above, glyphosate is generally considered to be a relatively safe herbicide.

Via the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate blocks the synthesis of phenylalanine-derived plant phenols, including lignin precursors and some classes of phytoalexins involved in plant resistance. It blocked the production of phaseollin in bean (*Phaseolus vulgaris* L.; Johal and Rahe 1988) and glyceollin in soybean (*Glycine max* (L.) Merr.; Holliday and Keen 1982). Sublethal doses of glyphosate inhibited the resistance mechanisms to *Phytophthora megasperma* Drechs. f.sp. *glycinae* in soybean (Keen *et al.* 1982), to *Colletotrichum lindemuthianum* (Sacc. and Magn.) Briosi and Cav. in beans (Johal and Rahe 1988) and to *Fusarium* spp. in tomatoes (Brammal and Higgins 1988). The production of lignin in asparagus (Salveit 1988) and flax (Sharma 1986), as well as phenolic compounds in roots of tomato seedlings (Brammall and Higgins 1988) were reduced by glyphosate. The fact that glyphosate can alter secondary compounds such as phytoalexins or lignin has several implications in a pest management prospective. This question related to glyphosate and to pesticides in general was reviewed by Lydon and Duke (1989).

Indirect effect

At the recommended rates, glyphosate is slow acting and rarely kills plants within 1 week after treatment. Glyphosate treated field bindweed took at least 3 weeks before starting to die (Duncan and Weller 1987) and volunteer potato plants took 1 month to die after being treated with glyphosate (Lamondia and Brodie 1986). Poor control was obtained by Belzile *et al.* (1987) during the first year but excellent control was achieved during the 2 subsequent years

when reduced doses (0.45-1.23 kg/ha) of glyphosate were used to control quackgrass (*Agropyron repens* (L.) Beauv.). These facts would be difficult to explain if one considers that glyphosate has only a direct phytotoxic effect through the inhibition of the shikimic acid pathway.

Johal and Rahe (1984) showed in beans that the herbicidal action of glyphosate was mediated by soil microorganisms. Bean seedlings growing in vermiculite or autoclaved soil survived a 10µg dose of glyphosate while the same dose killed plants grown in raw soil. 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. It was clearly demonstrated that microorganisms are the cause of the herbicidal efficacy of glyphosate.

Damage to crops

Lynch and Penn (1980) showed that rhizomes of quackgrass treated with glyphosate were readily colonized by *Fusarium culmorum* (W.G.Sm.) Sacc. and that an increase in the population of this fungus was associated with adverse effect on subsequently planted barley (*Hordeum vulgare* L.). A similar effect was observed by Mielke (1983) who reported an increased incidence of take-all (*Gaeumannomyces graminis* (Sacc.) von Arx and Olivier var. *tritici*) on wheat (*Triticum aestivum* L.) after quackgrass control with glyphosate. Lévesque *et al.* (1987) showed that glyphosate treatment of weed cover was associated with an increase in population of *Fusarium* spp. in both weed roots and soil. No damage on subsequently planted crops was observed.

The following observations are qualitative but suggest a correlation between glyphosate use and diseases. When radiata pine (*Pinus radiata* D.Don) was established in Melbourne in the mid sixties, there were no significant levels of root diseases. In the mid seventies, high levels of damping off and root rot caused by *Pythium* spp. and *Phytophthora cinnamomi* Rands, respectively, were present. Increased use of herbicides, including glyphosate, was suspected to be the cause of the new problem (Kassaby 1985; Kassaby and Hepworth 1987). In 1984 in Western Australia, there were substantial emergence problems in 30,000 ha of winter crops and glyphosate was the herbicide used in the majority of this area (Blowes 1987).

Objectives of research

The importance of microorganisms in the herbicidal action of glyphosate needs to be understood better in order to elucidate some of the problems described above. Very little has been done so far to understand this phenomenon and fundamental research is necessary for investigating with insight what is happening in the field.

The first objective of my research program was to determine if the differential sensitivity to glyphosate between bean seedlings grown in raw and heat treated organic soil as described by Johal and Rahe (1984) occurred with other plant species and soil types.

With the exception of Johal and Rahe (1984) and Lévesque *et al.* (1987), all publications dealing with fungal colonization of glyphosate treated plants

examined only a particular causal agent of a given disease. Hence, the second objective was to examine in detail the effect of glyphosate on the fungal colonization process in selected plant species, at different temperatures and soil moisture levels. The fungal colonization was examined both qualitatively and quantitatively.

The third objective was to examine the host and site (soil type) specificity of fungal colonizers of glyphosate treated plants and assess their pathogenicity in the absence of glyphosate.

CHAPTER II

The Effect of Heat Treatment of Soil on Efficacy of Glyphosate

The objective of this part of my research was to determine whether the differential sensitivity to glyphosate between bean seedlings grown in raw and autoclaved muck soil described by Johal and Rahe (1984) occurred in other plant species, grown in different soil types treated with another heat treatment technique. I also wanted to determine whether or not the heat treatment effect on glyphosate efficacy was related to a change in soil microflora.

MATERIALS AND METHODS

Soil sampling and storage.

Soil used in the various experiments was collected from field sites within 6 weeks prior to the start of the experiments. The four different soil types came from British Columbia and were: organic soil (muck) from a cultivated field in Cloverdale, mineral soil (silt loam) from a cultivated field near Aldergrove, orchard and virgin sandy soils from the Agriculture Canada Research Station, Summerland. The soils were stored outside in the shade in plastic garbage containers of 100-L capacity. The matric potential of a collected soil was adjusted to approximately -1.0 bar by air drying and then it was passed through a 4.5 mm sieve.

Soil autoclaving, microwaving and soil matric potential

Soil samples were heat-treated in either an autoclave or microwave oven. Autoclaving was done twice on consecutive days by holding bags containing 3 kg of soil at 1 bar, 121°C, for 1 h. Autoclaved bags were kept sealed until seeding which was done at least 1 week after autoclaving. Microwaving was done by placing a bag containing 2 kg of soil on a rotating plate for 6 min in a microwave oven at 560 watts (Ferriss 1984). The percent moisture at field capacity of the different soils was estimated with a 15 bar ceramic plate extractor (Soil Moisture Equipment Co.) using 1.0 bar plates to equilibrate samples at 0.1 bar pressure (Appendix I).

Seeds and rhizomes

Seeds of Northstar winter wheat, Topcrop beans, Sunnyvee corn (*Zea mays* L.) and Florida Broadleaf mustard greens (*Brassica juncea* (L.) Czern. and Coss.) were obtained from Buckerfield's, Ltd., Abbotsford, British Columbia. Apple (*Malus domestica* Borkh.) seeds of McIntosh parentage were obtained from Agriculture Canada Research Station, Summerland, British Columbia, and were scarified by storing them in wet sand at 4°C for 2 months until a few seeds started to germinate. Scarified apple seeds and all other seeds were surface sterilized by placing them for 2 min in a 1% NaOCl solution agitated by a stirring magnet and then rinsed thoroughly with sterile distilled water. Quackgrass rhizomes were collected from a local garden site just before their use in experiments. They were washed with tap water, cut to segments with single

buds, and segments that weighed 0.2 to 0.3 g were selected. They were left in the agitated 1% NaOCl solution for 4 min. The surface sterilized rhizomes were aseptically transferred onto the surface of sterile, water-saturated vermiculite in trays covered with aluminum foil for germination which took 1 week. The seeds were sown in 1.5 cm deep depressions in the surface of the soil made with a sterile glass rod. The germinated rhizomes were transferred to soil in holes made with a a sterile glass plate (5 x 0.4 cm) and subsequently covered. Sterile forceps were used to handle the seeds and rhizomes.

Glyphosate treatment

Roundup was used to treat the plants. Control plants received equivalent treatments with distilled water. The herbicide was applied using a Hamilton micromanipulator with 25 or 50 µl syringes. All plants growing in the same pot received the same treatment. Plants were treated 2 weeks after seeding except for quackgrass and apple seedlings that were treated 3 and 10 weeks after transplanting to the soil, respectively.

There were three different types of experiments: determination of LD_{50}'s, validation of LD_{50} estimates, and confirmation of the role of microorganisms in glyphosate herbicidal efficacy. There were four experiments for determination of LD_{50}'s, each analyzed independently. In the first experiment, bean and corn seedlings received 0, 5, 10, 25 and 100 µg of glyphosate applied as a single 1 µl droplet placed on the hypocotyl just below the cotyledons for beans and close to the sheath on the lowest leaf blade for corn. In the second experiment, quackgrass seedlings received 0, 5, 10 and 25 µg of glyphosate applied as two

0.5 µl droplets on each of the two youngest leaves. In the third experiment, wheat seedlings received 0, 1, 5 and 25 µg of glyphosate applied as three 1 µl droplets on the second youngest leaf and one on the youngest leaf. In the fourth experiment , apple seedlings received 0, 36, 72 and 144 µg of glyphosate contained in 0, 5, 10 and 20 droplets of 1 µl, respectively, and distributed on the five youngest leaves. For validation of LD_{50} estimates, bean and wheat seedlings were given a dose of glyphosate intermediate between the LD_{50} estimates for raw and heat treated soils (10µg and 1µg for bean and wheat seedlings, respectively). Glyphosate was applied as two 1 µl droplets placed on each of the two youngest leaves in beans and applied as above for wheat. For verification of the role of microorganisms, bean and wheat seedlings received 10 and 2.5 µg of glyphosate, respectively, and 0 µg for controls applied as described in the previous experiment.

To try to correct for differences in plant size, the stem diameter of each seedling was measured with a caliper (± 0.05 mm) just before glyphosate treatment. The diameter was measured perpendicularly to the axis formed by the first leaf or first two opposite leaves, 1 cm above the soil line for the monocotyledonous plants and just below cotyledons for the dicotyledonous plants.

Growth conditions

Plants were grown in plastic pots or trays. If not new, pots and trays were thoroughly washed with soapy water, rinsed, and soaked overnight in a solution of approximately 0.1% NaOCl.

In the first LD_{50} experiment, bean and corn seeds were planted in 16x12 cm trays at 18 seeds per tray. There was only one tray for each treatment combination. In the second experiment, four quackgrass germinating rhizomes were placed in each 10 cm pot. There were five pots for each treatment combination. In the third experiment, wheat seeds were planted in 10 cm pots, with four seeds per pot and two replicate pots. In the fourth experiment , two germinating apple seeds were planted in small polystyrene coffee cups and thinned to one seedling per cup immediately after emergence to give a total of 20 plants per treatment combination. For validation of LD_{50} estimates, bean and wheat seeds were individually placed in 4x3-cm trays for a total of 20 plants per treatment. For confirmation of the role of microorganisms, bean and wheat seeds were planted individually as in the apple experiment.

The plants were grown at a 16:8 h, day:night photoperiod and 25:19±1°C. Light intensity at plant height was 200±50 μEin m^{-2} s^{-1}.

Watering

All plants grown in trays or pots were watered to saturation daily during the period 3 days before to 1 week after glyphosate treatment, and otherwise every second day. Pots were raised to avoid contamination between them by leaching water. The soil matric potential for plants grown in coffee cups was adjusted to -0.1 bar daily during the period 3 days before to 1 week after glyphosate treatment, and otherwise every second day. For details of water adjustment technique see next chapter.

Inoculation of autoclaved soil with soil extract

For confirmation of the role of microorganisms, a 1:4 soil:water suspension was treated in one of three ways, namely (i) passed through four layers of cheese cloth (raw), (ii) autoclaved for 20 min in 1-L bottles or (iii) filtered by passing twice through two layers of 15-cm #1 Whatman filter paper and once simultaneously through a glass fiber filter and a 0.22-µm pore size Falcon millipore filter. Fifteen-ml quantities of each of these soil extracts were added 5 days before and at the time of glyphosate treatment to the surface of the soil.

Assessment of death of plants

The effect of treatments was recorded as proportion of dead plants 1 month after glyphosate treatments. Plants with a dead main stem but with new shoots were rated as alive.

Dilution plating

For estimating microbial populations in soil, one sample of approximately 20 g was taken from each of three replicate bags or pots. One half of a sample was used for estimating the percent dry weight of the soil and the other half was used for dilution plating. Microbial populations were expressed as CFU (colony forming units) per g dry weight of soil. Water extract samples were evaluated in triplicate by dilution plating and were recorded as CFU per ml of extract. Three replicate plates were made for each soil and water extract sample. Selective

media were used to estimate *Pythium* spp. (Mircetich 1971) and *Fusarium* spp. (Nash and Snyder 1962). Peptone dextrose rose bengal (Johnson and Curl 1972) was used for fungi and potato dextrose agar (PDA) for bacteria. The soil samples were first mixed with 100 ml of sterile distilled water; the soil water extracts were used directly. For *Pythium* and *Fusarium* spp., the plating was done by serial dilution (Lévesque *et al.* 1987) and by using a Spiral plater. For fungi and bacteria only the Spiral plater was used.

Statistical analysis

The plant mortality data were analyzed using GLIM and stepwise logistic regression (Baker and Nelder 1978). Variables were included in the model if their significance determined by the deviance drop was below 0.15 and rejected if above 0.15. In logistic regression, a regression type structure (e.g. $Y_i = \mu + \beta W_i + \alpha X_i$) is attributed to the "logit" $Y_i = \ln[\pi_i/(1-\pi_i)]$, where π_i is the true, or theoretical, proportion of dead plants in an entire conceptual population of plants (of which the data sample is assumed random, or representative). For a fixed W, as a function of X_i, the graph of π_i is S-shaped, the particular location and sharpness of the S depending on α and μ. Independent variables were the dose of glyphosate $X_i = \ln (dose_i + 1)$, and depending on the experiment, the centered value of the stem diameter $W_i = \ln diameter_i - \overline{\ln diameter}$, the type of soil, and the type of soil heat treatment. The last two factors were appropriately coded as categorical variables. In experiments where available space permitted, plant species were mixed randomly, coded as categorical variable and a single analysis was performed. When plant species had to be separated,

an independent analysis was performed for each species. The significance of the soil heat treatment effect on the dose-mortality response was given by the deviance drop obtained by fitting separate slopes to the different heat treatment instead of only one slope for all treatments.

LD_{50}'s, i.e., $\exp(X_{50}) - 1$, were estimated by setting the regression expression for Y_i equal to zero and solving for X_{50} (when the proportion π_i = 0.50, the logit $Y_i = 0.0$). The resulting expression for X_{50} was used to estimate X_{50} by substituting the fitted regression coefficients and the covariate values. As reliability assessment for these estimated X_{50} values, standard errors (S.E.) were calculated using the "Propagation of Errors" formula for approximate variance of a transformed variable (Rice 1987).The same formula was used to calculate the S.E.'s for proportions of dead plants in experiment verifying the contribution of microorganisms to herbicidal efficacy. The resulting fitted LD_{50}'s and their upper and lower confidence limits (plus or minus one S.E.) were obtained by applying the exponential function $LD_{50} = \exp(X_{50}) - 1$ to the symmetric upper and lower limits for X_{50}.

The data from dilution plating experiments were analyzed using a log-linear model with "Offset" values set at the dilutions used (Baker and Nelder 1978). The "Propagation of Errors" formula was used to calculate S.E.'s of estimated mean CFU values (Rice 1987).

RESULTS

Effect of heat treatment of soils on microbial populations

Autoclaving or microwaving of mineral or organic soils reduced the populations of *Pythium* and *Fusarium* spp. below the detection limits of the techniques used (Table 1). Except for *Fusarium* spp. in the organic microwaved soil, these two species failed to recolonize the heat treated soils to detectable populations by 14 days after exposure of the soils to sources of reinfestation, at which time glyphosate was applied to the seedlings. Two days after exposure of soil to sources of reinfestation, the number of CFU of fungi was 100 to 10,000 times lower in heat treated than in raw soil. Fourteen days after exposure to sources of reinfestation, fungal populations as estimated by dilution plating were lower in the heat treated than in the raw soils. Bacterial populations remained unchanged or varied only by one order of magnitude (Table 1).

Effect of glyphosate

It took more glyphosate to kill corn or bean seedlings growing in heat treated mineral soil compared to seedlings growing in raw mineral soil (Fig. 1). The effect was observed in both autoclaved and microwaved soils. No LD_{50}'s could be estimated for beans grown in microwaved soil since less than 50% of the seedlings died at the highest dose tested.

Table 1. Residual effect of autoclave and microwave treatments of mineral and organic soils on populations of various components of soil microflora 2 and 14 days after seeding the treated and raw soils with wheat. The heat treated soils were exposed to sources of reinfestation at seeding.

TIME	SOIL		CFU [a] (Standard Error) OF VARIOUS MICROFLORA COMPONENTS			
DAYS	TYPE	TREATMENT	*Pythium* spp.	*Fusarium* spp.	FUNGI	BACTERIA
2	MINERAL	RAW	$2.30(0.25)\times10^{3}$	$1.42(0.10)\times10^{4}$	$1.46(0.19)\times10^{5}$	$1.30(0.35)\times10^{7}$
2	MINERAL	AUTOCLAVE	<2[b]	<2	<65	$1.65(0.30)\times10^{7}$
2	MINERAL	MICROWAVE	<2	<2	$1.22(0.50)\times10^{3}$	$1.26(0.34)\times10^{6}$
2	ORGANIC	RAW	$4.90(0.43)\times10^{3}$	$1.87(0.13)\times10^{4}$	$1.37(0.18)\times10^{5}$	$7.35(3.07)\times10^{6}$
2	ORGANIC	AUTOCLAVE	<2	<2	$2.18(0.68)\times10^{3}$	$1.17(0.38)\times10^{6}$
2	ORGANIC	MICROWAVE	<2	<2	$1.09(0.52)\times10^{3}$	$2.45(0.53)\times10^{6}$
14	MINERAL	RAW	$2.12(0.27)\times10^{3}$	$2.56(0.20)\times10^{4}$	$1.76(0.24)\times10^{5}$	$1.49(0.32)\times10^{7}$
14	MINERAL	AUTOCLAVE	<2	<2	$1.74(1.85)\times10^{2}$	$1.19(0.31)\times10^{7}$
14	MINERAL	MICROWAVE	<2	<2	$3.70(0.83)\times10^{3}$	$4.49(1.12)\times10^{7}$
14	ORGANIC	RAW	$1.35(0.21)\times10^{3}$	$4.01(0.26)\times10^{4}$	$1.16(0.16)\times10^{5}$	$1.03(0.25)\times10^{7}$
14	ORGANIC	AUTOCLAVE	<2	<2	$7.77(1.01)\times10^{4}$	$1.21(0.30)\times10^{7}$
14	ORGANIC	MICROWAVE	<2	$1.22(0.29)\times10^{2}$	$3.78(0.91)\times10^{3}$	$8.26(1.48)\times10^{7}$

[a] Colony forming units per g dry weight of soil estimated by dilution plating.
[b] The "<" values represent the minimum CFU detectable by the technique used.

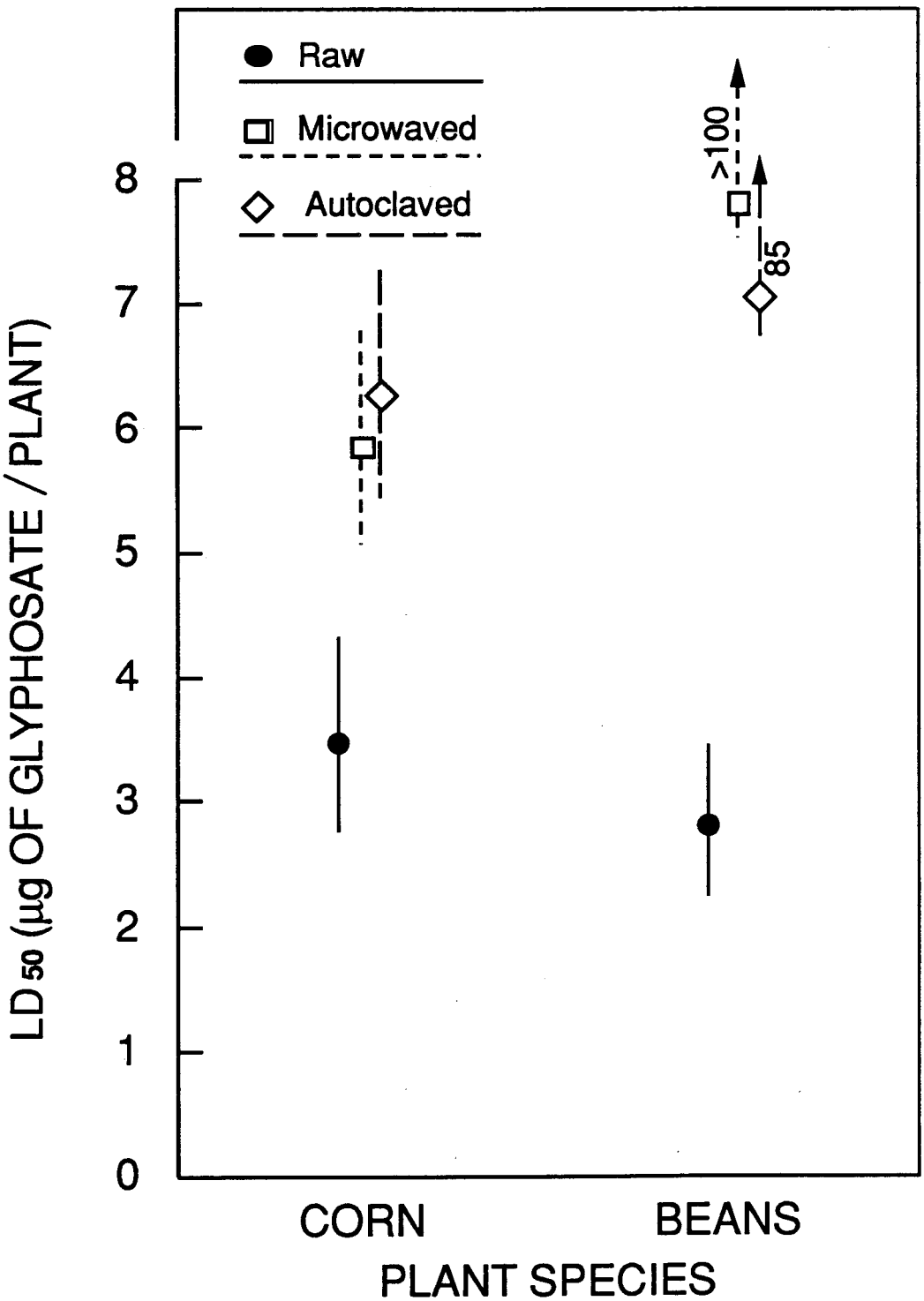


Figure 1. Effect of heat treatment of soil on LD_{50} values of corn and bean seedlings treated with glyphosate. Mineral soil was used and three treatments were compared: raw, microwaved and autoclaved soils. Vertical lines indicate standard error of estimates.

The stem diameter effect

The inclusion in the model of the explanatory variable stem diameter significantly improved the accuracy of the model fit, and so was included in the regression model. Using quackgrass as an example, the stem diameter tended to be slightly larger in quackgrass seedlings grown in microwaved soil than in quackgrass seedlings grown in raw soil, although the difference was not significant. The inclusion of stem diameter as a covariate in the analysis substantially reduced the standard errors (Fig. 2). The difference between LD_{50}'s of seedlings grown in raw soil and LD_{50}'s of seedlings grown in heat treated soil tended to be smaller with the inclusion of the covariate but the difference remained significant ($p < 0.05$).

The effect of soil type

It took more glyphosate to kill wheat seedlings growing in heat treated organic and mineral soils than in the respective raw soils, but the LD_{50} values were not affected by the soil type (Fig.3). However, in apple seedlings, differential sensitivity between heat treated and raw soils was observed in the apple orchard soil but not in virgin soil (Fig. 4).

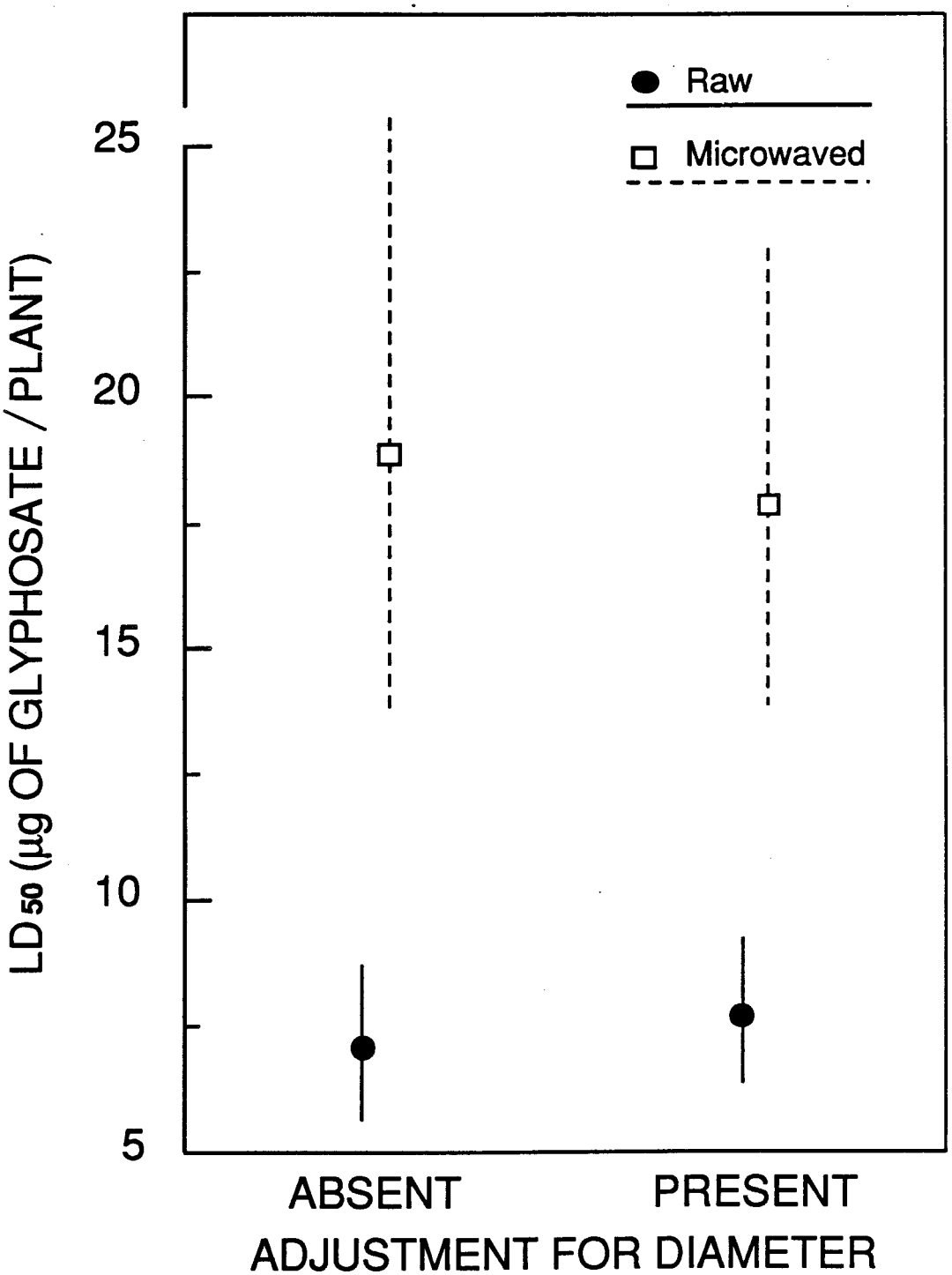


Figure 2. Effect of including the covariate, stem diameter, in analysis for estimating LD_{50} values for glyphosate on quackgrass seedlings grown in raw and microwaved organic soil. Vertical lines indicate standard error of estimates.

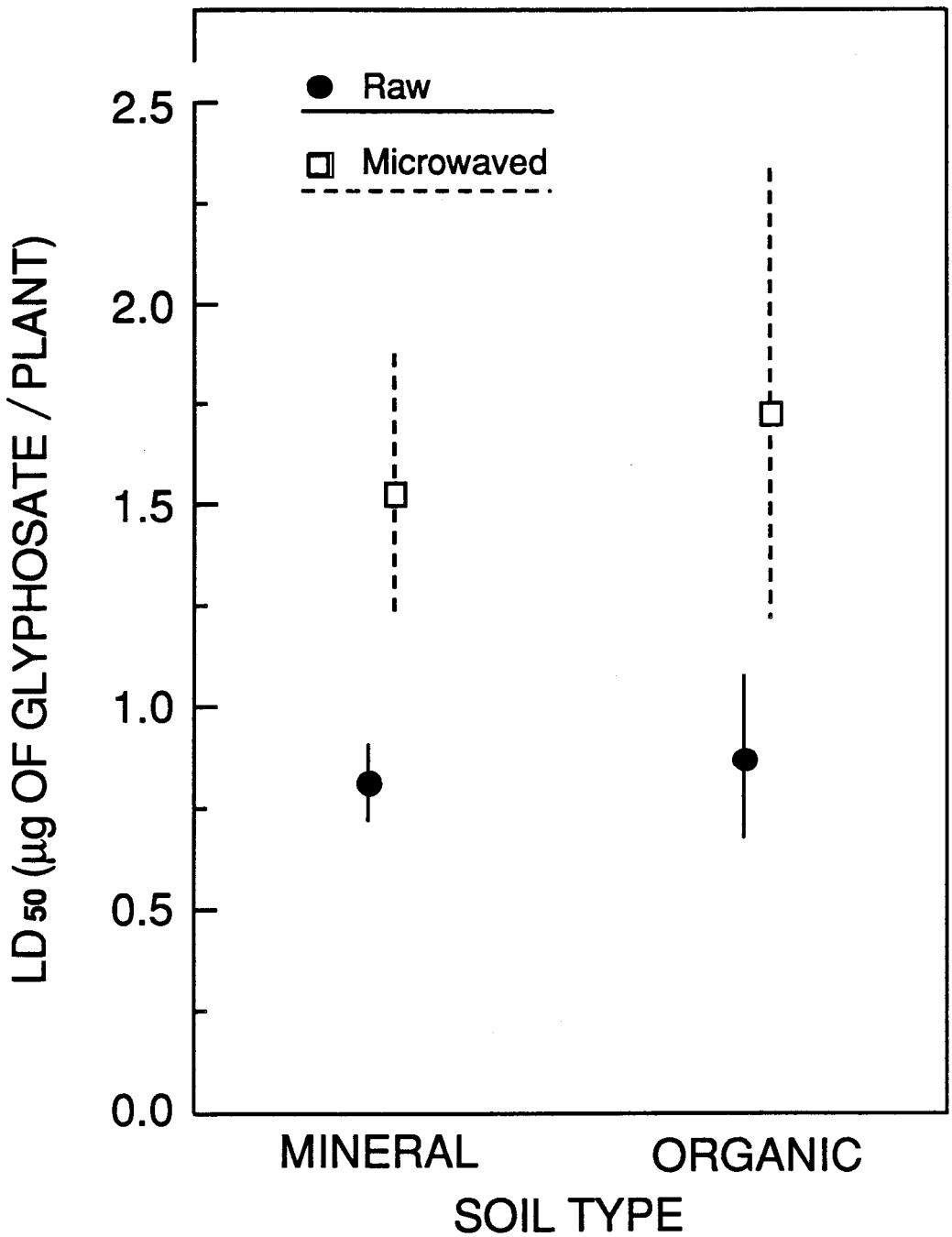


Figure 3. Effect of different soil types on LD_{50} values and on the differential sensitivity to glyphosate of wheat seedlings grown in raw and microwaved soils. Vertical lines indicate standard error of estimates.

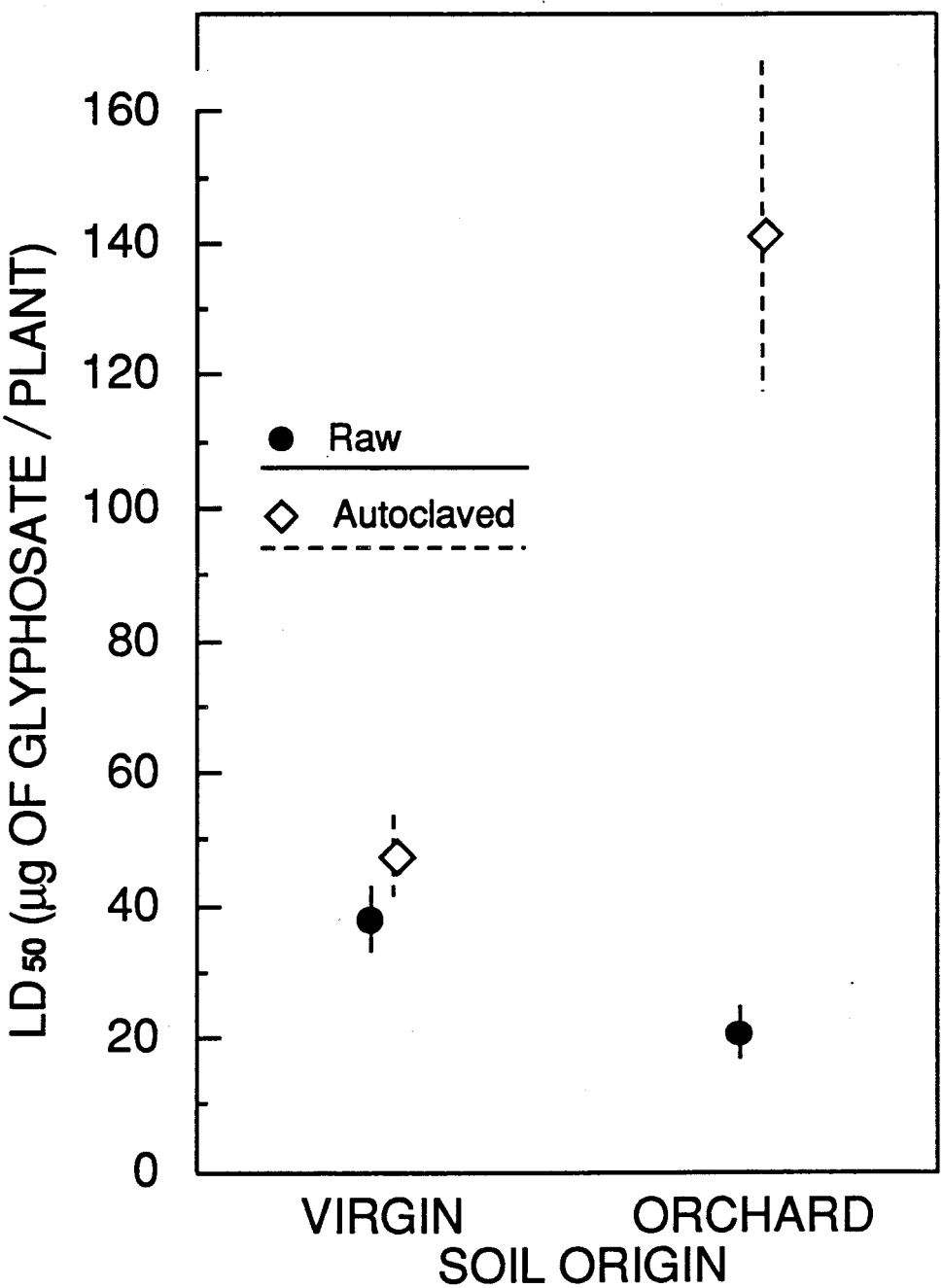


Figure 4. Effect of different soil origins on LD_{50} values and on the differential sensitivity to glyphosate of apple seedlings growing in raw and autoclaved soils. Vertical lines indicate standard error of estimates.

Testing accuracy of LD_{50} estimates

All bean seedlings growing in raw organic or mineral soils died after receiving 10 µg of glyphosate but less than 20% died in heat treated soils after receiving the same dose (Fig. 5). In wheat , the same trend was observed except that the mortality in seedlings grown in untreated soil ranged from 60 to 80% after receiving 1 µg of glyphosate (Fig.5). Soil heat treatment significantly modified the magnitude of the differential effect but soil type had no effect.

Microbial effect

Raw water extracts had high concentrations of propagules of *Pythium* spp., *Fusarium* spp., fungi and bacteria (Table 2). Autoclaving or microwaving reduced *Pythium* and *Fusarium* spp. propagule numbers in these extracts to below detection levels. Populations of bacteria and fungi were reduced at least 10,000 fold (Table 2). *Pythium* spp. were reestablished in mineral soil by amendment of the soil with raw water extracts and were present at 2% of the population observed in raw soil 6 days after the first amendment (Tables 1 and 2). *Pythium* spp. were not detected in the organic soil after addition of raw water extract. Amendment with raw extracts raised numbers of *Fusarium* spp. propagules in mineral and organic autoclaved soils to 2% and 20%, respectively, of what they were in raw soil (Table 1 and 2). *Pythium* and *Fusarium* spp. were not detected in soils treated with autoclaved or filtered extracts.

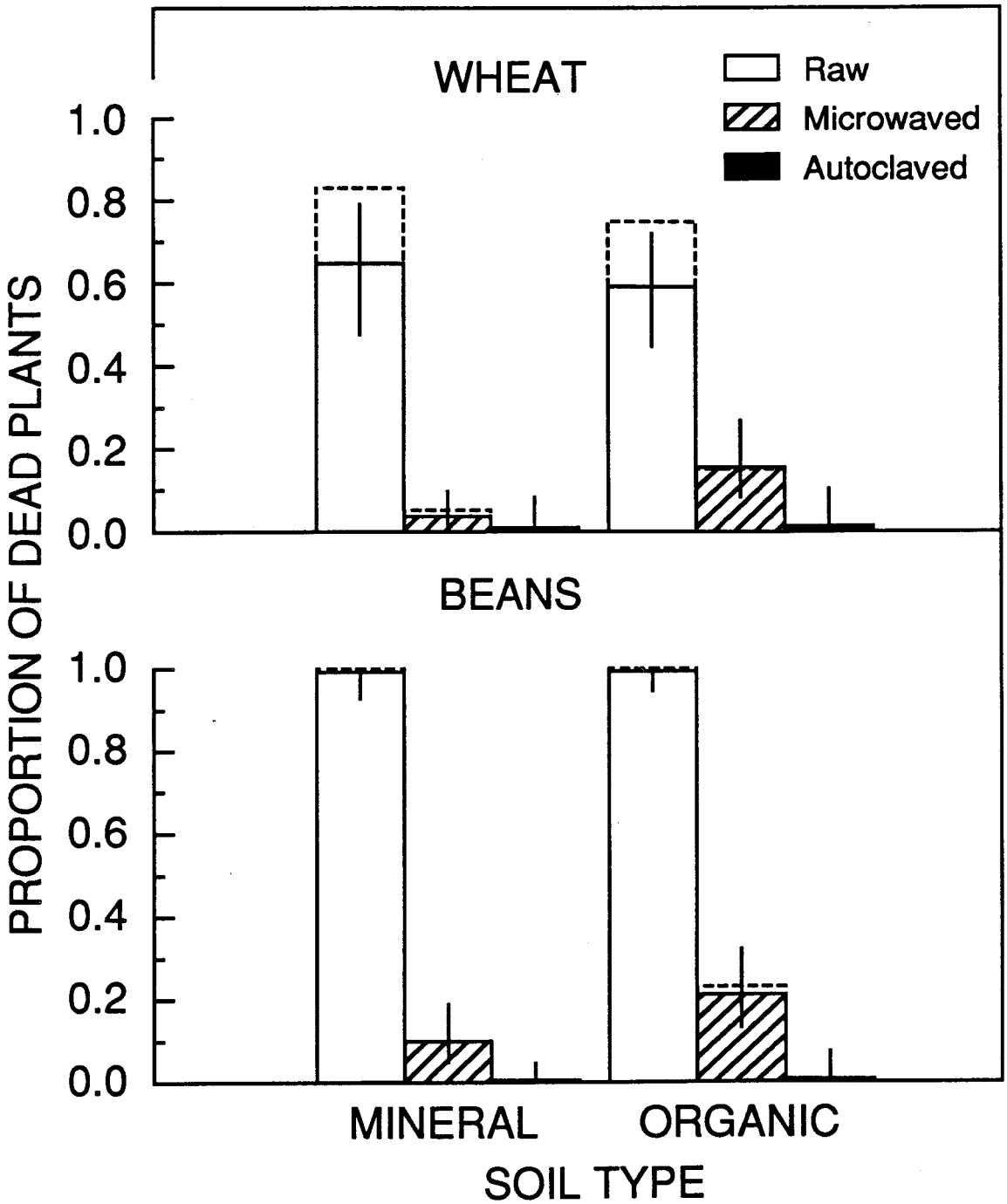


Figure 5. Effect of soil type and heat treatment on mortality, expressed as proportions of dead seedlings in test populations of wheat and bean seedlings treated with 1 and 10 µg of glyphosate, respectively. Solid bars represent values adjusted to an average stem diameter and dotted lines represent the observed proportions. The vertical lines indicate the standard error of the proportions.

Table 2. Relative populations of various components of soil microflora with standard errors (S.E.) in raw, filtered and autoclaved extracts from mineral (M) and organic (O) soils and in the autoclaved soils after adding the various extracts. Fifteen ml of extracts were added 5 days prior to and at the time of glyphosate treatment. Assessment of CFU in soil was done 1 day after the last amendment.

SAMPLE TYPE	EXTRACT TYPE	EXTRACT TREATMENT	CFU [a] (S. E.) OF VARIOUS MICROFLORA COMPONENTS *Pythium* spp.	*Fusarium* spp.	FUNGI	BACTERIA
EXTRACT	M	RAW	$6.83(0.39)\times10^{2}$	$1.54(0.06)\times10^{3}$	$2.08(0.20)\times10^{4}$	$1.14(0.15)\times10^{6}$
EXTRACT	M	FILTRATION[b]	<2[c]	<2	<2	$1.31(1.56)\times10^{1}$
EXTRACT	M	AUTOCLAVE	<2	<2	<2	$2.09(0.62)\times10^{2}$
EXTRACT	O	RAW	$4.95(0.33)\times10^{3}$	$1.06(0.05)\times10^{3}$	$1.69(0.18)\times10^{4}$	$8.39(0.13)\times10^{5}$
EXTRACT	O	FILTRATION	<2	<2	<2	$4.36(2.85)\times10^{1}$
EXTRACT	O	AUTOCLAVE	<2	$4.44(3.14)\times10^{0}$	$2.11(6.09)\times10^{0}$	$8.71(4.02)\times10^{1}$
SOIL	M	RAW	$6.48(0.76)\times10^{1}$	$5.38(1.13)\times10^{2}$	$3.44(1.61)\times10^{3}$	$8.29(1.09)\times10^{6}$
SOIL	M	FILTRATION	<1	<1	$5.71(6.51)\times10^{2}$	$7.65(1.06)\times10^{6}$
SOIL	M	AUTOCLAVE	<1	<1	$8.61(24.9)\times10^{1}$	$9.44(1.31)\times10^{6}$
SOIL	O	RAW	<1	$8.35(0.70)\times10^{3}$	$2.72(0.50)\times10^{4}$	$1.35(0.18)\times10^{7}$
SOIL	O	FILTRATION	<1	<1	$2.22(0.44)\times10^{4}$	$2.67(0.33)\times10^{7}$
SOIL	O	AUTOCLAVE	<1	<1	$1.73(0.38)\times10^{4}$	$6.78(0.95)\times10^{6}$

[a] Colony forming units per ml of extract or per g dry weight of soil, estimated by dilution plating.
[b] Through 0.2µm millipore filter.
[c] The "<" values represent the minimum CFU detectable by the technique used.

Table 3. Effect of soil extracts added to autoclaved mineral and organic soils on % mortality (Standard Error) of glyphosate treated[a] wheat and bean seedlings (based on 20 plants).

SOIL TYPE	PLANT SPECIES	% DEATH FOR DIFFERENT TREATMENTS RAW	FILTERED[b]	AUTOCLAVED
MINERAL	WHEAT	25.0 (9.68)	0.0	0.0
MINERAL	BEANS	100.	0.0	0.0
ORGANIC	WHEAT	5.00 (4.88)	0.0	0.0
ORGANIC	BEANS	5.00 (4.88)	0.0	0.0

[a] All plants that received no glyphosate survived, even in soils that received the different soil extracts.
[b] Through 0.2µm millipore filter.

In mineral soil, the herbicidal efficacy of glyphosate on bean seedlings was fully restored by the addition of raw soil water extract and partly re-established in wheat seedlings (Table 3). The mortality of glyphosate treated plants grown in autoclaved organic soil amended with raw soil extract was not significantly different than zero. Herbicidal efficacy was not recovered in any of the soils to which autoclaved or filtered soil water extracts were added (Table 3).

DISCUSSION

It takes more glyphosate to kill seedlings grown in heat treated soil than in raw soil. The effect of heat treatment of soil on the herbicidal efficacy of glyphosate observed by Johal and Rahe (1984) on beans grown on muck soil has been verified for different plant species of the monocotyledonae, and dicotyledonae, both herbaceous or woody. It was observed for different soil types that had been autoclaved or microwaved. Tworkoski and Sterret (1988) and Mugnier (1988) showed that plants grown hydroponically were fairly insensitive to glyphosate. There was no death but a 30% weight reduction was reported in winter wheat that received a low dose of 0.095 kg/ha (Ruiter *et al.* 1988). The wheat plants were grown in sand and humic potting soil but no mention was made about pasteurization. Unfortunately, in the majority of the recent publications dealing with glyphosate applied to plants grown in pots containing at least a portion of natural soil, the presence or absence of any soil sterilization is not specified. My results show that it is not reasonable to

compare data concerning effects of glyphosate obtained from pot trials unless the soil and its treatment is fully described.

Differential sensitivity to glyphosate between seedlings grown in raw and heat treated soils was due to a change in microflora. Soil microflora was changed both qualitatively and quantitatively by heat treatment. Moreover, when raw soil water extracts were added to heat treated soil, herbicidal efficacy was restored in soil where *Pythium* spp. were re-established (Tables 2 and 3). It does not prove that *Pythium* spp. were the cause of the recovery of efficacy, but certainly shows that recovery of efficacy cannot be accomplished by any random recontaminant since the fungal populations in the soil where efficacy was not restored were within one order of magnitude of natural levels (Tables 1 and 2). The fact that differential sensitivity to glyphosate between heat treated and raw soil was observed in apple seedlings grown in orchard soil but not in virgin soil also supports the conclusion that not all fungi can act as synergists of the glyphosate herbicidal action (Fig. 4). Mugnier (1988) associated a greater sensitivity to glyphosate in roots of plants transformed by *Agrobacterium rhizogenes* (Riker.) Conn. with an increase in translocation to the roots due to a sink effect. It is possible that certain fungi increase the sink effect of the roots by inducing a defense response by the host. However, this phenomenon would not explain the antidote-like effect of metalaxyl to plants growing in the presence of *Pythium* spp. (Johal and Rahe 1984). By either mechanism, increased translocation because of sink effect or death of plant after fungal attack, microorganisms can play a synergistic role in the herbicidal efficacy of glyphosate at certain dose levels.

In the experiment performed to test the accuracy of LD_{50} estimates, the doses of 1 and 10µg for wheat and beans, respectively, were selected to optimize the differential sensitivity to glyphosate between seedlings grown in heat treated and raw soils. It worked exactly as predicted and validates the statistical model used for the analysis. Logistic regression was shown to have more power than other forms of regression analysis commonly used for proportions (Appendix II). The validation of the LD_{50} also shows that the slightly different methods of application of glyphosate did not affect the estimates. Boerboom and Wise (1988) showed that glyphosate applied on Canada thistle (*Cirsium arvense* (L.) Scop.) at given high doses does not translocate as well from droplets of small volume as from droplets of large volume. At the dose levels and droplet volume used in the majority of my experiments, no significant differences in translocation were observed.

In quackgrass, the doses of glyphosate applied, and at which differential effects were observed, fall within the range of the high label-recommended rates for Roundup (*i.e.* 2.5 kg of glyphosate per ha). Assuming that all the applied herbicide comes in contact with the quackgrass plants, it represents a field dose of 312 µg of glyphosate per g of dry weight of quackgrass biomass based upon an average quantity of 8 T of total dry biomass of quackgrass per ha (Ohman and Kommedahl 1964; Werner and Rioux 1977). At the time of glyphosate treatment, the average total dry weight of the seedlings was 0.053 g (S.E. 0.008). The doses ranged from 0 to 25 µg of glyphosate per plant which represents 0 to 470 µg of glyphosate per g dry weight. These doses go beyond the high label-recommended rate of application. Using the same transformation

as above, the LD_{50} observed in heat-treated soil becomes 340 µg of glyphosate per g of dry weight of quackgrass biomass, which is very close to the high label-recommended dose of 312 µg. Quantitative experiments were done by Harvey and Crothers (1988) using flax treated with glyphosate that had been mixed with a dye in order to find how much glyphosate ultimately reached the plant. They showed that glyphosate applied at a field dose of 1.44 kg/ha represented an average dose of 132 µg per g dry weight or 22 µg per shoot. The dose was as low as 5 µg per shoot or 29 µg per g dry weight because of variations in spray patterns. They considered that these minima would be even lower in commercial fields since the plant stand and spray droplets distribution would be much less uniform than at the experimental station. These low doses are within the range of the LD_{50}'s I estimated. It is likely that microorganisms play a synergistic role in the herbicidal efficacy of glyphosate on quackgrass in the areas of the fields that receive low doses because of the lack of uniformity of herbicide spray application.

CHAPTER II

Fungal Colonization of Glyphosate Treated Plants

Two widely different plant species and soil types were used in these experiments. The dicotyledonous plant, *Phaseolus vulgaris*, was selected for this study on fungal colonization because of the amount of information already available on the effect of glyphosate on resistance of beans (Johal and Rahe 1984, 1988) and the high tolerance of beans to glyphosate when grown in heat treated soil (Chapter I). *Triticum aestivum* L. was selected as a representative of the monocotyledonous plants because it is very sensitive to glyphosate. Mineral (silt loam) and organic (muck) soils were selected because they have been used in previous experiments dealing with glyphosate and microflora, and because they represent two very different soil types available in our geographical area.

The first objective was to develop a standard technique for counting the different fungal colonizers of roots. I wanted to develop a more reliable technique than the standard one of plating randomly selected root pieces. Although extensively utilized, this root plating technique gives a bias toward colonizers of larger segments of the root system as researchers tend to select the bigger pieces for plating, and it is difficult to interpret the results in terms of colonizers per plant.

To further improve the estimates obtained from this new root plating technique, I investigated the possibility of using stem dry weight for adjusting the CFU per root system for differences in root size. Ideally root weight or root

surface should have been used as adjustment factors but these values are not available for technical reasons. Therefore, I had to establish the effect of glyphosate on root dry weight and, if this effect were negligible for the period under study, examine the correlation between root and stem dry weight. If the correlation was good, stem dry weight could be used as a covariate to adjust CFU values for root size.

The second objective was to determine the most important fungal genera colonizing glyphosate treated wheat and bean seedlings grown in the two different soil types.

The third objective was to investigate the effect of temperature and soil moisture on fungal colonization of wheat and bean seedlings grown in the mineral soil.

MATERIALS AND METHODS

Soil sampling and storage

Soils used in the various experiments were collected from field sites within 4 months of the start of the experiments. Two soil types were used: a mineral soil (silt loam) from a cultivated field near Aldergrove, British Columbia, an organic soil (muck) from a cultivated field in Cloverdale, British Columbia. The soils were stored in 100-L plastic garbage containers outside in a shaded area or in a greenhouse under benches. The matric potential of a collected soil was adjusted to approximately -1.0 bar by air drying and then the soil was passed through a 4.5-mm sieve.

Method of herbicide application

Wheat and bean seedlings received 2.5 µg and 10 µg, respectively, of glyphosate 12 days after seeding. The dose was applied in four 1-µl droplets, three to the second youngest leaves and one to the youngest for wheat and two droplets on each of the two youngest leaves for beans. In the experiments dealing with effect of environmental variables, glyphosate was applied as Roundup (glyphosate) and control treatments received water. In the other experiments, Rodeo (glyphosate) was used with 0.1% surfactant and surfactant alone was used for control treatments.

Root plating technique

Intact root systems were removed from bean or wheat seedlings grown in small polystyrene coffee cups. Roots longer that 7 cm had curled at the bottom of the cup and were excised. Root systems were washed on a 0.6-mm sieve using tap water under pressure. The roots were excised by cutting the stems immediately above the crown. A 125-mm autoclaved #1 Whatman filter paper was placed on top of a 150-mm filter paper in a Buchner funnel. The funnel was filled with approximately 250 ml of a 1% NaOCl solution. A three-way valve was placed in sequence after the funnel connecting into a vacuum source (1 bar). Using the valve, one could deviate the vacuum source from the funnel and block the outflow for filling the funnel or, alternatively, could connect the funnel with the vacuum source for quick disposal of the sterilizing solution. Each root system was individually floated for 2 min during which time the roots were evenly spread with a set of sterile forceps. Forceps were left leaning on the side

of the funnel to hold the floating roots in place. After the 2-min sterilization time, the solution was quickly withdrawn and the roots were washed with sterile distilled water whilst under suction. The 125-mm filter paper with the adhering root system was then inverted onto a 150-mm Petri dish of agar culture medium. The paper and the root system were pressed onto the agar with a sterile, bent glass rod. A small quantity of sterile distilled water was squirted onto the paper to cause the release of the root system. The paper was then removed leaving the root system evenly spread on the agar.

Roots of seedlings were plated immediately before, 2 and 4 days after , as well as 6 or 7 days after glyphosate treatment. There were always four to five replicate plants per treatment at each sampling time except for the first experiment with beans when there were three replicates.

Method for estimating CFU per root system

Potato dextrose agar (PDA) was used as a general culture medium and selective media were used to estimate *Pythium* spp. (Mircetich 1971) and *Fusarium* spp. (Nash and Snyder 1962).

Roots were examined daily under a dissecting scope and emerging colony boundaries were circled on the bottom of the dish with a felt pen of a different color every day. Colonies were subcultured when genus of a fungus was uncertain.

Stem-root dry weight correlations

Entire root systems were washed as described above. The stem, the distal portion of a root system normally excised for CFU estimates, and the proximal part used for CFU estimates, were dried at 80°C for 48 h and weighed individually to ±0.1 mg .

Plant growth conditions

Seeds of Northstar winter wheat and Topcrop beans were surface sterilized in 1% NaOCl for 2 min and aseptically transferred to the surface of sterile and water saturated vermiculite in a tray which was then covered with aluminum foil. After 24 h at 25°C, the seeds were planted in holes of 1.5 cm depth made with a glass rod in small polystyrene coffee cups containing 100-130 g of soil. An equal amount of soil (± 0.2 %) was put in each cup for each experiment and soil type. Soil dry weight as percent of total weight was estimated so that the amount of dry soil in each cup was known.

All plants were grown under 250 μEin m^{-2} s^{-1} at a 16:8 h day:night light regime. When no temperature treatments were involved, the plants were grown at a 25:18°C, day:night regime. All plants were contained in one growth chamber. For the series of experiments with two temperature levels, the plants were grown at a 21:21°C regime until 3 days before glyphosate treatment when temperature was set at either a 17:17°C or 25:25°C regime. Two growth chambers were utilized for each temperature level.

Watering

The moisture content measured as percent by weight of the mineral and organic soils was estimated for samples equilibrated in a 15 bar ceramic plate extractor (Soil Moisture Equipment Co.) using 1.0 bar ceramic plates for 0.1, 0.33 and 1.0 bar pressures and 15 bar plates for 3.0 and 15 bar pressures (Appendix I). At least three replicate soil samples were taken for each moisture estimate. A Basic language program with built in equations for the retention curves was made for a Tandy Model 100 portable computer interfaced with a Mettler digital balance. For each given experiment, the amount of soil dry weight per pot was known and included in the program. The pots were put on the scale and the amount of water needed to bring the matric potential to a given level was displayed on the screen. The appropriate amount of water was measured and applied with an automatically refilled 20-ml glass syringe with plastic tubing attached to a reservoir of distilled water.

When soil moisture was not a treatment by itself, the soil matric potential was adjusted to -0.06 bar every second day and daily starting 3 days before glyphosate treatment. Two matric potentials were selected to investigate the effect of soil moisture: -0.06 and -1.0 bar. All the cups were watered every 2 days to -0.25 bar until 3 days before glyphosate treatment when they were adjusted daily to the two selected treatment levels. For the high matric potential of -0.06 bar, the pots never became drier than -0.3 bar and for the low matric potential of -1.0 bar, they never became drier than -3.0 bar.

Effect of heat treatment of soil on glyphosate efficacy

Wheat seedlings grown at the two selected temperature and moisture regimes and grown in raw and autoclaved mineral soil were treated with glyphosate as described for CFU estimate experiments. There were eight replicate plants for each treatment combination. Plant mortality was estimated 2 weeks after treatment.

Statistical analysis

The root dry weight and CFU on beans data were analyzed using GLIM and log linear models (Baker and Nelder 1978). Significance was determined by the drop in deviance when adding the variable tested to the model. CFU values on wheat roots reached a maximum before the last plating so log linear models gave a poor fit. In order to solve that problem, the wheat data were fitted to the following logistic model for the theoretical mean (CFU), under a given set of conditions:

$$\text{MEAN (CFU)} = \frac{\mu}{1 + e^{\alpha_i + \beta_{ijkl} t}}$$

where t is the time after glyphosate treatment, μ is the maximum density of CFU on root systems for both *Pythium* spp. and *Fusarium* spp., α determines the intercept at time equal to zero for fungus *i*, and ß is a measure of rate of growth of fungus *i*, herbicide treatment *j*, and if applicable, temperature *k* and soil moisture *l*. BMDP3R software was used to estimate the parameters using a case weight of 1/mean (Jennrich 1983). Significance was evaluated by

estimating the F-ratio between the model with and without the variable of interest. In the experiment using four different environmental conditions, the maximum rates of colonization given by $-\mu\beta/4$, which can be described as the slope at the inflexion point of the S-shape curve given by the model, were estimated in order to simplify data presentation (Seber and Wild 1989). The associated standard errors were calculated with the "propagation of errors" formula (Rice 1987).

RESULTS

Effect of glyphosate on root weight.

Glyphosate significantly reduced root growth as measured by dry weight in both wheat and bean seedlings grown in mineral and organic soils during the 6-day period following treatment (Fig. 6). The roots of glyphosate treated bean seedlings lost weight during the 6-day period and the weight of wheat roots remained constant.

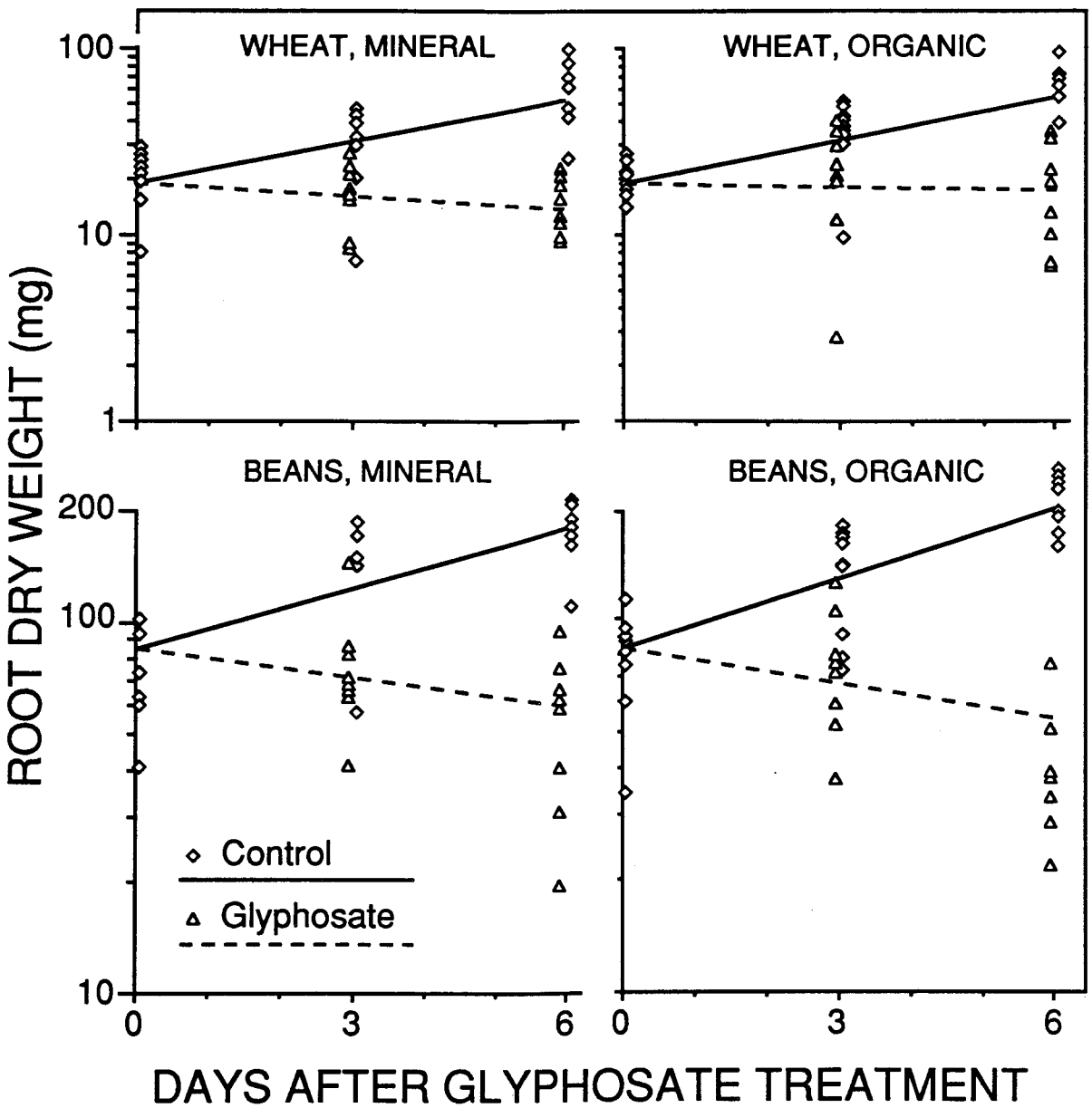


Figure 6. Effect of glyphosate on root dry weight of 2-week old wheat and bean seedlings grown in raw mineral and organic soils as a function of days after glyphosate treatment.

Colonization of wheat seedlings.

Of all the 243 isolates recovered on PDA from the roots of glyphosate treated wheat seedlings grown at a 25:18°C day:night regime, 65% belonged to *Pythium* spp. and 33% to *Fusarium* spp. There was a higher rate of colonization in glyphosate treated plants compared with untreated plants for both *Pythium* spp. and *Fusarium* spp. in either mineral or organic soil (Fig. 7). An average of seven CFU's of *Pythium* spp. per plant was observed 2 days after glyphosate treatment of seedlings grown in mineral soil, whereas the root systems of untreated plants in the same experiment rarely (one observed in total of 16 plants) yielded *Pythium* spp.

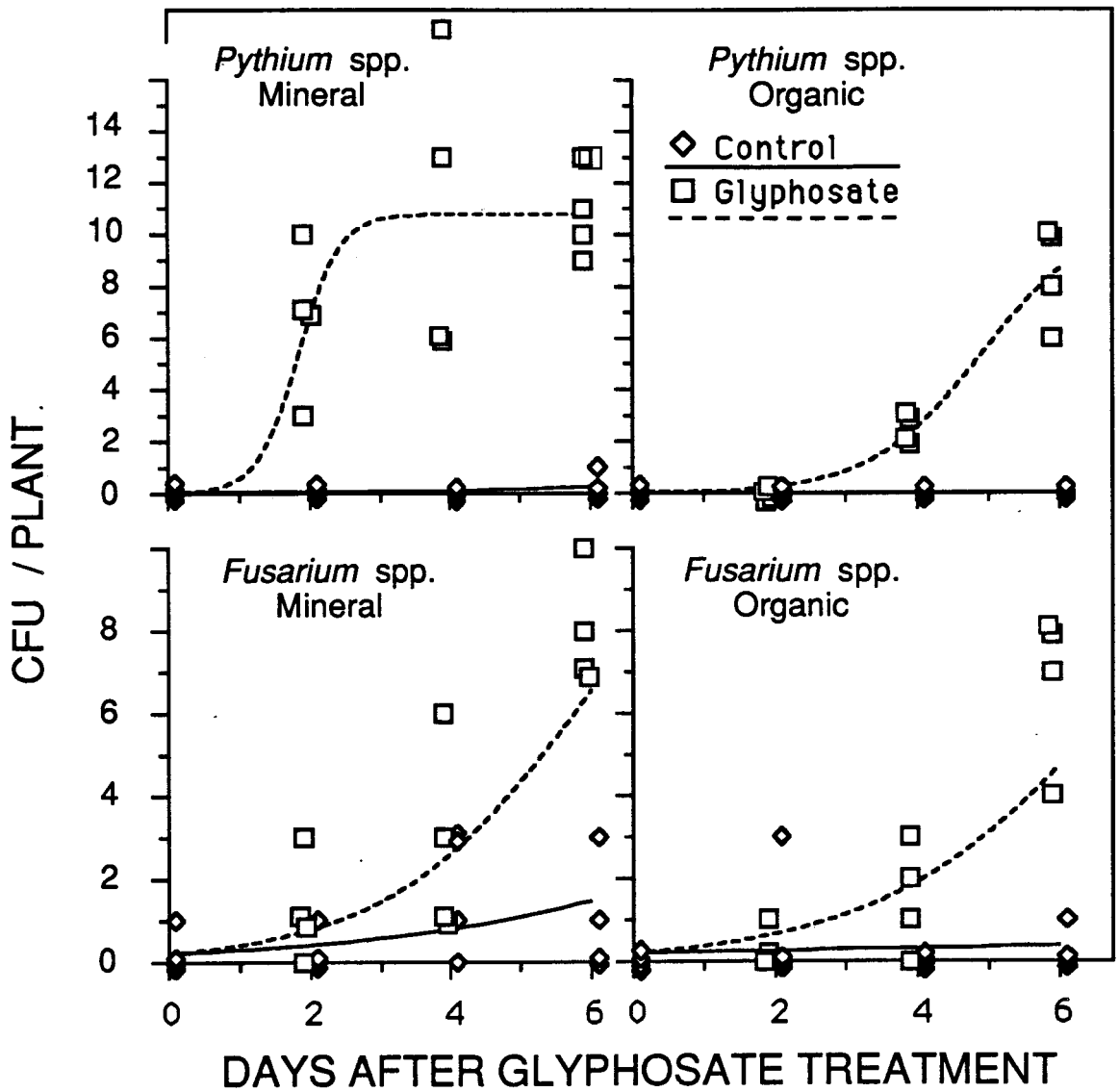


Figure 7. Effect of glyphosate and soil type on the number of colony forming units (CFU) of *Pythium* and *Fusarium* spp. per root system as a function of days after treatment of wheat seedlings with glyphosate.

Colonization of bean seedlings

Four and 6 days after glyphosate treatment, the roots of bean seedlings were in an advanced stage of deterioration. Typically, very little of the root system was left and the NaOCl presumably sterilized the macerated root system completely, as fungal growth came only from large rootlets close to the crown from what appeared to be one single actively growing colony. Therefore, only the data for 2 days after glyphosate treatment are described here. Of a total of 18 isolates recovered from bean seedlings, 15 belonged to *Pythium* spp. and 1 to *Fusarium* spp. There was an average of 2.5 CFU per root system in glyphosate treated seedlings, whereas control seedlings had no significant level of colonization by *Pythium* spp. Over the entire course of the experiment, only four *Pythium* spp. colonies were observed in a total of 24 untreated plants. Colonies emerging from the glyphosate treated bean roots did not develop from discrete circular points as in wheat, but from elongate regions of rootlets. Soil type had no effect on the colonization by *Pythium* spp. The overall colonization by *Fusarium* spp. was not significantly different from zero

Effects of temperature and soil moisture in wheat grown in mineral soil

Differential sensitivity in heat treated soil

Decreased sensitivity of wheat seedlings to glyphosate in heat treated soil, as described in the previous chapter, was observed over the range of imposed temperature and soil moisture variation. More than 87% of the wheat

seedlings growing in raw soil died in each of the four environmental regimes, whereas only 25% or less died when grown in autoclaved soil (Table 4).

Proportions of different colonizers

Two, 4 and 7 days after glyphosate treatment, *Pythium* spp. and *Fusarium* spp. were the most frequent colonizers on roots of glyphosate treated wheat seedlings plated on PDA (Table 5). In the four different environmental conditions, these two genera together comprised from 83 to 93% of a total of 1090 isolates obtained from 120 seedlings. *Pythium* spp. were more prevalent than *Fusarium* spp. The rates of colonization in *Pythium* spp. were exactly similar to that observed in the following experiment using Mircetich (1971) selective media and the rates by *Fusarium* spp. were lower than those found by using Nash and Snyder (1962) selective media. Since the experiment with selective media is believed to be more accurate, the details of the data obtained from root plating on PDA are not shown.

Rates of colonization by *Pythium* spp. and *Fusarium* spp. estimated using selective media

Two examples taken from the overall data analysis are given in Fig. 8. These represent the CFU on roots of glyphosate treated wheat seedlings grown at 25°C and -0.06 bar, the fitted logistic curves and the estimated maximum colonization rates.

Table 4. Effect of temperature, soil moisture and heat treatment on the number of wheat seedlings that died after receiving 2.5 μg of glyphosate. There was a total of eight plants for each treatment combination. Mortality was rated 14 days after application of glyphosate.

SOIL MOISTURE	TEMPERATURE	MORTALITY	
BAR	°C	RAW SOIL	AUTOCLAVED SOIL
-0.06	25	7/8	0/8
-0.06	17	8/8	1/8
-1.00	25	8/8	0/8
-1.00	17	8/8	2/8

Table 5. Effect of temperature and soil moisture on the relative importance of *Pythium* and *Fusarium* spp. as root colonizers, reported as percent of total number of CFU (N) observed on surface sterilized roots of glyphosate treated wheat seedlings that were plated on PDA. Combined data for roots harvested 2, 4 and 7 days after treatment with glyphosate are presented.

SOIL MOISTURE	TEMPERATURE	PERCENT OF TOTAL CFU		N
BAR	°C	*Pythium* spp.	*Fusarium* spp.	
-0.06	25	77	16	237
-0.06	17	78	4	309
-1.00	25	67	25	284
-1.00	17	74	17	260

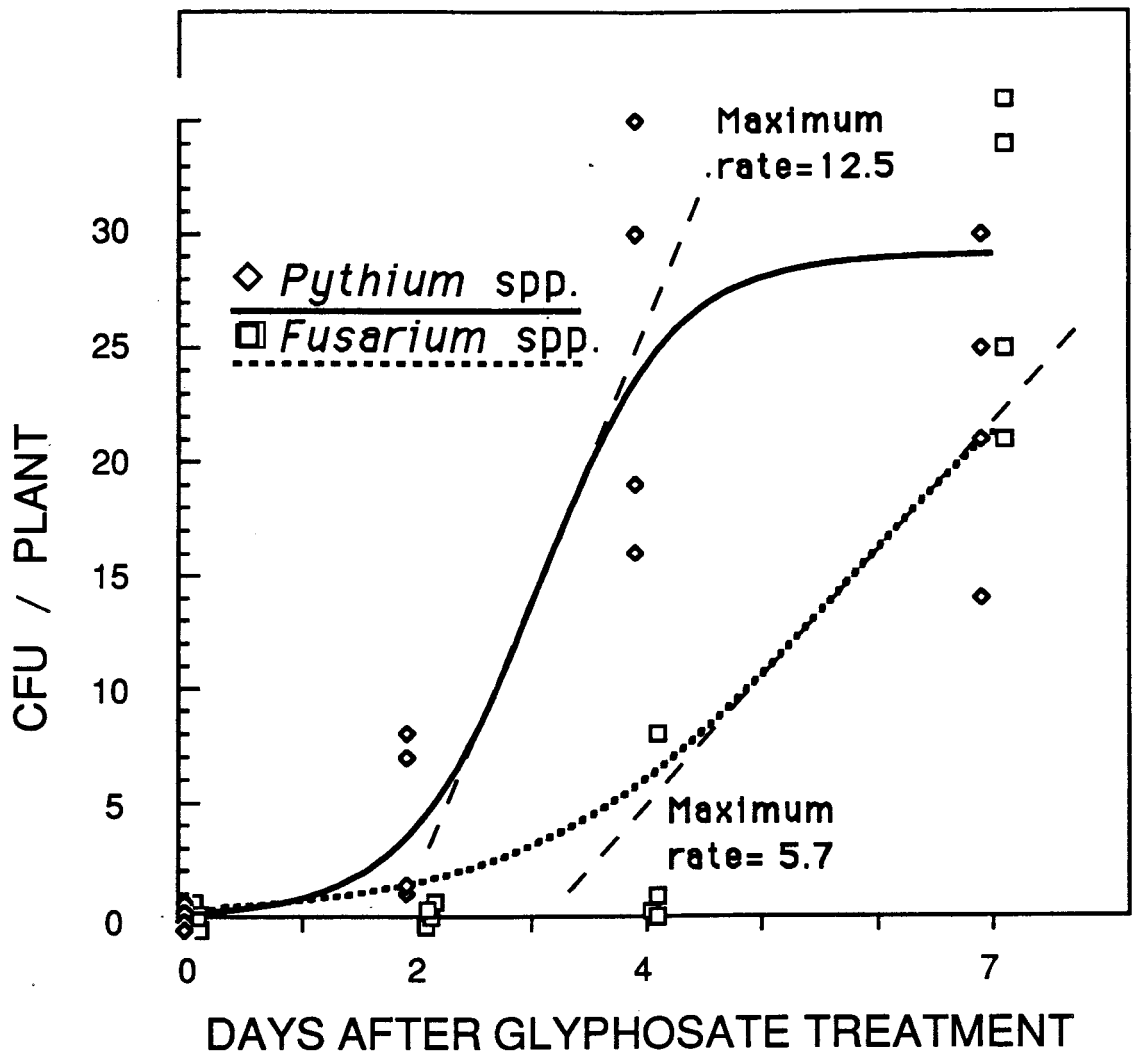


Figure 8. Colony forming units of *Pythium* and *Fusarium* spp. per root of glyphosate treated wheat seedlings as a function of days after glyphosate treatment. The maximum rates of colonization are represented by the dashed lines tangent to the logistic curves. Plants grown at 25°C and -0.06 bar, in raw mineral soil.

Pythium spp. were the predominant colonizers under the four environmental conditions tested (Fig. 9). The maximum colonization rates by *Fusarium* spp. in glyphosate treated seedlings were about half of the rates observed for *Pythium* spp. There was no significant colonization by *Pythium* spp. in plants not treated with glyphosate while colonization by *Fusarium* spp. in control seedlings was significant. Except for colonization by *Fusarium* spp. in the moist and cooler condition, there was always a significant increase in colonization due to glyphosate treatment.

Colonization rates in glyphosate treated seedlings by both fungal species were significantly reduced at the lower temperature. The magnitude of the reduction was significantly larger for *Pythium* spp. than for *Fusarium* spp. The colonization rates of glyphosate treated plants by *Fusarium* spp. were significantly higher in drier conditions whereas the tendency for lower colonization rates by *Pythium* spp. in drier conditions was not significant.

Site of colonization

Out of a total of 740 colonies of *Pythium* spp. emerging from roots of glyphosate treated plants plated on selective medium, 23% were identified as being derived from root tips. There was a total of 487 colonies of *Fusarium* spp. in roots plated on selective medium, 11% of which emerged from root tips. The number of colonies emerging from root tips was significantly higher for *Pythium* spp. than for *Fusarium* spp. (2 x 2 contingency table test).

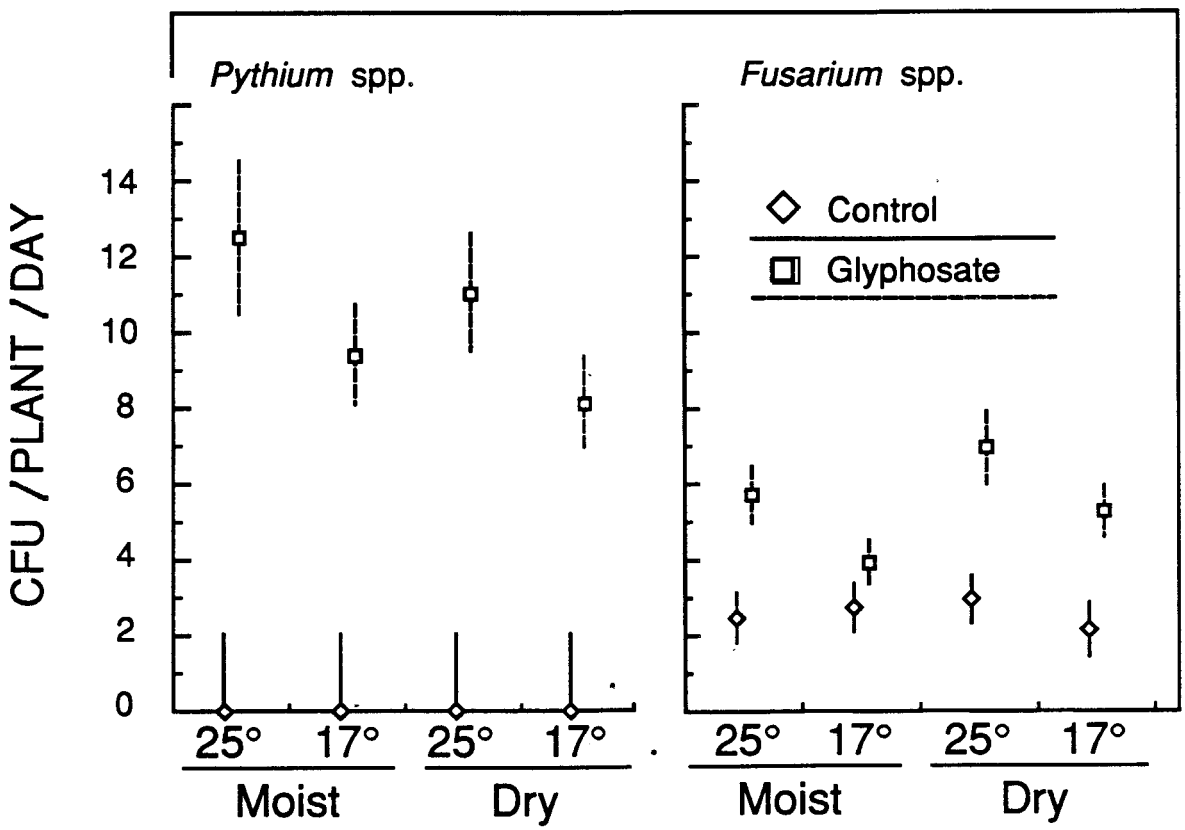


Figure 9. Effect of glyphosate, temperature and soil moisture on the maximum rates of colonization by *Pythium* and *Fusarium* spp. in roots of wheat seedlings grown in raw mireral soil, estimated by plating on selective media. The estimated maxima were derived from a logistic model which explained 85% of the variance and their standard errors are represented by the vertical lines.

Effects of temperature and soil moisture in beans grown in mineral soil

CFU's of *Pythium* and *Fusarium* spp. using selective media

Roots were plated on selective media 0, 1 and 2 days after glyphosate treatment. At day one, there was no significant increase in colonization by either *Pythium* spp. or *Fusarium* spp. due to glyphosate.

Two days after glyphosate treatment, the number of CFU of *Pythium* spp. per root system was significantly higher in glyphosate treated seedlings grown at 25°C compared to control seedlings in either dry or moist soil conditions (Fig. 10). In seedlings grown at 17°C, glyphosate had no significant effect but colonization in control seedlings was significant.

For *Fusarium* spp., there was a significant increase in colonization due to glyphosate in seedlings grown under dry conditions (Fig. 10). In seedlings grown under moist conditions, there was a significant colonization by *Fusarium* spp. in control plants but there were no significant increases due to glyphosate.

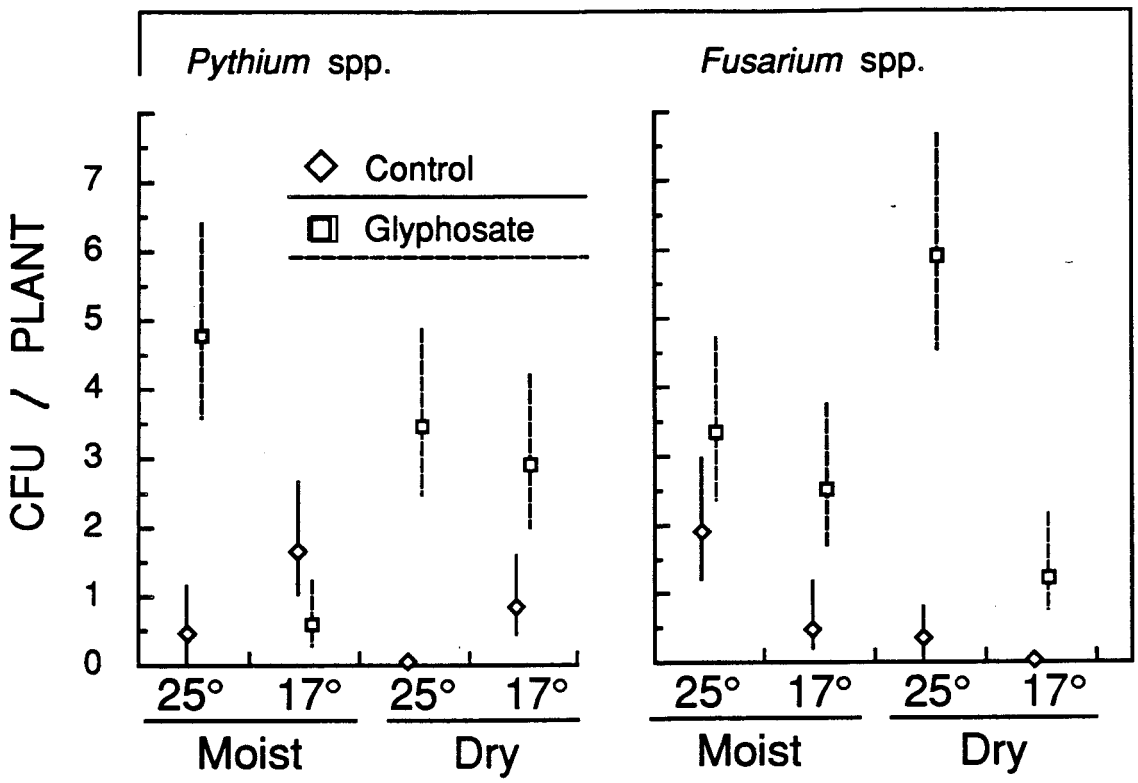


Figure 10. Effect of glyphosate, temperature and soil moisture on the number of colony forming units (CFU) of *Pythium* and *Fusarium* spp. on roots of bean seedlings grown in raw mineral soil, plated on selective media 2 days after glyphosate treatment. The vertical lines represent the standard errors of the average CFU's.

DISCUSSION

The root plating technique I developed gave valuable and reproducible results. The colonization rates by *Pythium* spp. in the experiments with wheat grown in mineral soil under different environmental conditions were exactly the same in two separate experiments where roots were either plated on PDA or Mircetich (1971) selective medium. The technique was successfully tried using a variety of plant species: apple, onion and alfalfa seedlings as well as part of the root system of apple trees (data not reported). This technique gently transforms what was an organized three dimensional structure into an evenly spread two dimensional one. It is easy to spread the roots uniformly while they are floating in the sterilizing solution. The removal by suction of the solution with the Buchner funnel does not change the disposition that was laid out during the sterilization time. The roots adhere well to the filter paper although the paper has to be kept more moist with stiffer roots like those of onions than for wheat. The technique is easier to perform with fibrous roots than with plants that have a tap root.

Information about what part of the root system is colonized can be gathered. Colonies arise as discrete circles along the rootlets and can be counted easily. When a rootlet is heavily infected, a single colony rapidly emerges from a long portion of the rootlet. The term CFU was used to describe the colonies because I do not know if discrete colonies represent different infection sites or different emergence loci of an internally growing fungus. This a very useful and precise technique (Lévesque and Rahe 1988), and I hope that the question of site of infection will be clarified in the near future.

Glyphosate treatment almost immediately stopped the root growth of both bean and wheat seedlings. Vaughn and Duke (1986) showed that roots of hydroponically grown soybean seedlings had arrested cell division 3 days after treatment. The rate of root growth of 1.5- to 2-month old Canada thistle plants decreased linearly with increasing dose of glyphosate (Carlson and Donald 1988). Kudoyarova *et al.* (1988) demonstrated that application of glyphosate to 3-day old corn seedlings reduced the levels of alcohol insoluble bound auxin. They hypothesized that this reduction of auxin is the cause of growth inhibition. The reduction in root biomass I observed in beans is likely attributed to microbial degradation of the root tissues. The roots of bean seedlings in all experiments were in an advanced stage of deterioration 4 and 6 days after treatment. Because of this drastic effect of glyphosate on root growth and the consequent effect on stem growth, stem dry weight could not be used to adjust CFU values per plant to a mean stem weight. Such adjustment would have increased the CFU values in glyphosate treated plants and decreased the values in control plants. I preferred to take the conservative approach and used the CFU values as they were observed on each root system to compare colonization between glyphosate treated and control plants even though correlations between stem and root dry weights in control plants were good (r^2 of 0.81 in wheat and 0.68 in beans). If this root plating is used in experiments where root size is not altered so drastically (e.g. comparing root colonization by a pathogen between two relatively resistant cultivars) or where the adjustment does not bias toward confirming the hypothesis, the stem dry weight could indeed be used to standardize the CFU values per plant. One other possible refinement would be to use an image digitizer to calculate the surface area of

the agar covered by the root system and obtain colonization data as CFU per unit area.

Roots of seedlings of wheat and beans were colonized by fungi within 2 days after glyphosate treatment. Glyphosate inhibits the growth of yeast by the same mechanism as occurs in higher plants, namely, the inactivation of 5-enolpyruvylshikimate-3-phosphate synthase (Bode *et al.* 1986). It inhibits the *in vitro* growth of many fungi (Harris and Grossbard 1979; Stedman 1982; Estok *et al.* 1989) including *Fusarium* spp. (Brown and Sharma 1984). It is very unlikely that glyphosate leaching from roots stimulated the fungi and increased colonization level. It is more probable that the seedlings became more susceptible to fungal colonization because of reduced capacity for synthesis of phenylalanine-derived phenolic compounds and a higher level of stress.

In glyphosate treated wheat and bean seedlings grown in organic and mineral soils, *Pythium* spp. were by far the most important fungal colonizers. These fungi, rarely present in root tissues of untreated plants, penetrated the roots within 2 days of herbicide application. It is rather surprising to find such an extensive colonization by a fungus that was rarely present in the tissues of untreated plants. It is possible that *Pythium* spp. had colonized root hairs or cells close to the epidermis of untreated plants and that the surface sterilization process killed these superficial colonizers. Most likely, the ability of *Pythium* spp. to grow rapidly helps it colonize a tissue that became suddenly less resistant to fungal attack. A high proportion of *Pythium* spp. colonies were found at the root tip probably because of a larger quantity of exudates present there. Root exudation is at its greatest level at root tips because of the thin and

unsuberized epidermal cell walls in this region (Cook and Baker 1983). Root tip exudation is possibly even greater than normal in treated plants because of the inhibition of cell division by glyphosate (Carlson and Donald 1988).

Fusarium spp. were the second most important group of colonizers of glyphosate treated plants and even though glyphosate treatment increased colonization in most instances, *Fusarium* spp. were present at substantially high populations in untreated plants, particularly in wheat seedlings. These localized infection sites would normally allow for some reproduction of the fungus but provide also a great advantage for colonizing the tissues of glyphosate treated plants. Thus, it was surprising to find that *Fusarium* spp. did not take full advantage of its pioneer colonist status, being outcompeted by *Pythium* spp. The increase in *Fusarium* CFU in glyphosate treated plants compared to control plants could be explained by the capability of *Fusarium* spp. to penetrate the roots and create new infection sites. Brammal and Higgins (1988) found that *Fusarium oxysporum* Schlecht. f.sp. *radicis-lycopersici* entered the epidermis of roots of resistant tomato cultivar 24 h after glyphosate treatment, penetrated the suberized hypodermis 48 h after treatment and the endodermis 96 h after. Hyphae were growing inter and intra cellularly.

Studying the colonization of different weed species in the field, Lévesque *et al.* (1987) reported that *Fusarium* spp. were by far the most important colonizers whereas in the present study *Pythium* spp. were the most important colonizers. Plant age would be one of the most likely causes for such a difference in results. Some of the weeds in the study of Lévesque *et al.* (1987) were perennials and the annuals were more than 3 weeks old, while seedlings

younger than 2 weeks old were used here. This would mean that a high proportion of the root tissues were suberized and less readily accessible to *Pythium* spp. in the former study. The root plating techniques used in the two studies were different : root pieces were plated by Lévesque*et al.* (1987) whereas entire root systems were used in the present study. The plating of root pieces excluded fine root tips, where I showed in the present study that *Pythium* spp. occurred preferentially to *Fusarium* spp. One other possibility was a difference in plant growth conditions. The study by Lévesque *et al.* (1987) was done in the field during a particularly hot and dry summer. Hot and dry conditions are the best for colonization of glyphosate treated plants by *Fusarium* spp (Figs. 9 and 10).

Colonization by either *Pythium* and *Fusarium* spp. was consistently higher in plants grown at 25°C compared to plants grown at 17°C. The two predominant *Pythium* spp. isolated from roots of bean and wheat seedlings treated with glyphosate (see next chapter) have their optimal growth rate at 25-30°C (Van der Plaats-Niterink 1981). In a field study, Hardman and Dick (1987) showed that temperature was more highly correlated with *Pythium* spp. propagule levels than was rainfall. *Fusarium* spp. causing wilt and all formae speciales of *F. oxysporum* have their optimal growth rate in culture around 28°C (Bruehl 1987). One of the best ways to find the optimal temperature for fungal colonization of root tissues is to determine for a range of temperatures the ratio of host and fungus growth rates (Leach 1947). The higher the ratio, the faster is the growth of the host in comparison to the growth of the pathogen. High colonization rates are likely to be encountered at temperatures where this ratio is low. Since roots stopped growing after glyphosate treatment, the optimal

temperature for colonization of glyphosate treated plants should be equivalent to their optimum for *in vitro* growth.

An alternative explanation for the difference in colonization at different temperatures could be that less glyphosate was translocated at the colder environmental condition. Masiunas and Weller (1988) found that less glyphosate, measured by radioactivity of ^{14}C-glyphosate, was translocated in potato plants (*Solanum tuberosum* L.) grown at a 13/4°C regime than at 24/13°C. There was four times less radioactivity in roots of plants grown in the colder condition. I believe that decreased translocation at the lower temperature in my experiments was negligible for several reasons. First, the difference between the temperatures used by Masiunas and Weller (1988) is much larger than that which I imposed. Second, the dose of 2.5µg for wheat was selected when I found that the dose of 1.0µg used in the previous chapter was not killing the plants grown in a new batch of soil collected at the same site. I conducted a small experiment using the same potting, glyphosate application and growth conditions (25/18°C) as that used in the present study in order to determine the appropriate dose to use for the experiments described in this chapter. Each plant of each group of five to seven wheat seedlings grown in raw soil received 0.0, 1.0, 1.5, 2.0, or 3.0 µg of glyphosate and 0, 0, 14, 17, and 100%, respectively, of the treated seedlings died. It was later shown that a dose of 2.5µg could kill almost all plants grown in any of the imposed environmental conditions (Table 4). If less glyphosate had been translocated at 17°C, these plants would not have died. Thirdly, the three plants out of 32 that died in the autoclaved soil were the ones grown at the cooler temperature showing a

reversed trend than that which should have been observed had the glyphosate been translocated better at 25°C than at 17°C (Table 4).

Colonization by *Fusarium* spp. was enhanced by a decrease in moisture in both bean and wheat seedlings. In cool and wet conditions *Fusarium spp.* did not colonize glyphosate treated plants more than control plants (Figs. 9 and 10). There was a trend toward higher colonization by *Pythium* spp. in wetter conditions but it was not significant ($p \geq 0.05$). *Pythium* spp. tend to cause more problems on crops that have readily accessible water while *Fusarium* spp. cause more disease under drier conditions (Cook and Baker 1983). *Fusarium* spp. behaved according to this theory but *Pythium* spp. were little affected by the different moisture levels tested. The soil matric potential of -1.0 bar in the drier condition was selected because control bean seedlings showed symptoms of wilting 24 h after adjustment to any matric potential drier than -1.0 bar. It is possible that in drier conditions, colonization by *Pythium* spp. could have been reduced. It is also conceivable that glyphosate treatment of the plants causes the roots to leach creating a microenvironment in the rhizosphere favorable to *Pythium* spp.

The soils used in these experiments were natural raw soils with a very diverse microflora. The fact that *Pythium* spp. were the predominant colonists in all the four imposed environmental conditions showed that growth of competitors or antagonists was never enhanced enough to restrict the colonization by *Pythium* spp.

I expected to find differences among fungi at the generic level between the colonizers of glyphosate treated bean and wheat seedlings. No qualitative

differences in colonizers were found between the two plant species and soil types. *Pythium* spp. are the most important colonizers of glyphosate treated bean and wheat seedlings grown in muck and loam soil. If there is specificity in these colonizers, it will be at the species or subspecies level.

CHAPTER III

Site, Host Specificity and Pathogenicity of Fungal Colonizers

The questions of host specificity and pathogenicity of fungal colonizers of glyphosate treated plants is of tremendous importance. If the same fungal colonizers are found in different plant species, *i.e.*, there is no host specificity, and if the colonizers can be pathogenic without the use of glyphosate, the mechanism of herbicide damage in the field after glyphosate application could be investigated with some important new fundamental knowledge about the overall mode of action of glyphosate. If host specificity does exist, the risks for fungal damage on subsequently planted crops is likely to be more restricted. So far, I have not found specificity in fungal colonizers of glyphosate treated plants at the generic level.

The first objective of this last part of my research was to compare at the DNA level *Pythium* isolates recovered from glyphosate treated bean and wheat seedlings grown in either mineral or organic soils. The *Pythium* genus was selected because it is the most important primary colonizer of glyphosate treated bean and wheat seedlings. The use of restriction fragment length differences to differentiate between and within species of organisms was recommended by Rose *et al.* (1982). Curran *et al.* (1986) were able to differentiate between isolates of *Meloidogyne incognita* (Kofoid and White) Chitwood using restriction fragment length differences of repeated DNA sequences, and Curran and Webster (1987) differentiated isolates within species using hybridization with labelled DNA probes. Formae speciales and

races of *Fusarium* spp. could be differentiated with the same techniques (Manicom *et al.* 1987; Coddington *et al.* 1987). These two techniques were selected for this analysis at the DNA level. Preliminary investigation was done using eight isolates in order to find the proper DNA extraction procedure, restriction enzymes and DNA probes. The resulting procedure was then applied to a new set of 53 isolates.

The second objective was to investigate *in vivo* the pathogenicity and host specialization of some isolates selected on the basis of the grouping after DNA characterization and cluster analysis.

MATERIALS AND METHODS

Soil sampling and storage

Soils used in the various experiments were collected from field sites within 4 months prior to the beginning of the experiments. Two soil types were used: the mineral soil (silt loam) came from a cultivated field near Aldergrove, British Columbia, the organic soil (muck) from a cultivated field in Cloverdale, British Columbia. The soils were stored in 100-L plastic garbage containers outside in a shaded area. The matric potential of a collected soil was adjusted to approximately -1.0 bar by air drying and then the soil was passed through a 4.5-mm sieve.

Method of herbicide application

Wheat and bean seedlings received 2.5 µg and 10 µg, respectively, of glyphosate 12 days after seeding. The dose was applied in four 1-µl droplets,

three to the second youngest leaves and one to the youngest for wheat and two droplets on each of the two youngest leaves for beans. Glyphosate was applied as Roundup and control treatments received water.

Plant growth conditions

Seeds of Northstar winter wheat and Topcrop beans were surface sterilized in 1% NaOCl for 2 min and aseptically transferred on the surface of sterile and water saturated vermiculite in a tray which was then covered with aluminum foil. The next day, the seeds were put in holes 1.5 cm deep made with a glass rod. Plants were grown in small polystyrene coffee cups filled with 140-150 g of mineral or organic soil. An equal amount of soil (± 0.2 %) was put in each cup for each soil type. Soil dry weight as percent of total weight was estimated so the amount of dry soil in each cup was known.

All plants were grown under 250 μEin m^{-2} s^{-1} at a 16:8 h day:night light and 25:18°C day:night temperature regime. All plants were contained in one growth chamber.

Root plating technique

The root plating technique described in the previous chapter was used and 2 days after glyphosate treatments, wheat or bean seedlings grown in either mineral or organic soils were plated on *Pythium* or *Fusarium* selective media (Mircetich 1971, Nash and Snyder 1962). There were five replicate plants per treatment combination.

Recovery of Isolates

A collection of eight *Pythium* isolates was used to develop a technique to differentiate isolates at the DNA level. These isolates came from the first experiments on fungal colonization of wheat and bean seedlings described in the previous chapter. They were isolated from glyphosate treated bean and wheat seedlings grown in either mineral or organic soils . There were two *Pythium* isolates from each soil type-plant species treatment combination. They were obtained from roots plated 2 days after glyphosate treatment except for wheat in mineral soil where roots were plated 4 days after glyphosate treatment. These isolates were representative of all isolates and were sent to the Biosystematics Research Center, Agriculture Canada, Ottawa, for identification by Dr. D.J.S. Barr.

A more extensive collection of *Pythium* spp. isolates was made from a new set of plants grown and treated with the technique explained in this chapter. Three *Pythium* spp. isolates were taken from each of the five replicate plants. When more than three isolates were present on a root system, the isolates were taken from different rootlets as far from each other as possible. In wheat seedlings grown in organic soil, there were a few plants that had fewer than three isolates per root system. A total of 53 isolates, 23 from wheat and 30 from beans, was obtained and used for DNA analysis.

Culture of *Pythium* spp.

Hyphal tips were transferred from colonies emerging from root systems to *Pythium* selective medium (Mircetich 1971). The *Pythium* spp. isolates were grown successively on the selective medium, then on water agar with streptomycin and penicillin (50 and 100 units/ml, respectively), before transfer to a broth medium. V-8 broth without cholesterol was prepared following the technique described by Ayers and Lumsden (1975) except that when the mycelium was grown for DNA extraction, the broth was filtered successively through a 15-cm #1 Whatman filter paper, a 7.5-cm Whatman grade A glass fiber filter, and a 0.22-μm Falcon millipore filter before autoclaving. Agar plugs of 8 mm diameter were taken from the margins of colonies on water agar to inoculate the broth medium. For DNA extraction, the isolates were grown in 50 ml of broth in 250-ml Erlenmeyer flasks. For pathogenicity studies, the isolates were grown in 200 ml of broth in 500-ml flasks. Flasks were put in the dark at 26±1°C and incubated for 9 to 10 days. After the incubation time, mycelium and broth samples were examined under the microscope to make sure that there was no bacterial contamination.

Mycelium extraction and storage

The contents of a culture flask were poured into an 8-cm Buchner funnel, and the broth was removed by suction. The mycelium was rinsed with cold sterile 0.1M NaCl solution. Free liquid was sucked out and the mycelium was put in a sterile 15-ml polypropylene disposable tube. Protease buffer (0.1 M tris buffer pH 8.0, 0.05 M Na_2EDTA, 0.2 M NaCl, and 1.0% sodium dodecyl

sulphate) and proteinase K (BRL, 1 mg/ml of buffer) were added at the rate of 4-6 ml for each g of sucked dry mycelium. The mycelial suspension was frozen right away in liquid nitrogen and either extracted immediately or stored at -70°C until processing.

Mycelial lysis and DNA extraction procedure

The following procedure is a modified version of Curran *et al* (1986). Polypropylene tubes frozen in liquid nitrogen were broken open and the contents ground with a pestle and a mortar cooled in liquid nitrogen. The mortar was put on a hot plate at its lowest setting until the material had thawed and the resulting liquid had become very viscous. The suspension was extracted once with half a volume of redistilled phenol (pH 8.0) saturated with TE buffer (0.01M tris buffer pH 8.0, 0.001 M Na_2EDTA) and centrifuged at 5000 rpm for 5 min. The interphase was extracted, transferred to another 15-ml tube, and mixed gently with half its volume of TE buffer. This mixture was extracted with phenol as mentioned before and the supernatant was mixed with the supernatant from the first extraction. The combined aqueous fractions were extracted with phenol twice more and then with chloroform-isoamyl alcohol (24:1) until the interphase was clear. DNA in the last supernatant was precipitated by adding 2.5 volumes of 95% cold ethanol. The solution was centrifuged for 5 min at 5000 rpm, the pellet washed twice with 70% ethanol, air dried, and resuspended in 400 µl of TE buffer. RNA was digested by adding 10 µg per ml of DNase-free RNase and incubating at 37°C for 30 min. NaCl concentration in the solution was brought up to 0.2 M by adding the appropriate amount of 4.0 M NaCl, and the DNA was precipitated as previously described. The pellet was air dried and resuspended

in 50-100 µl of TE buffer. Approximately 50 µg of total DNA were obtained from 0.5 g of mycelium. Diphenylamine reaction (Ashwell 1957) was used to estimate the amount of DNA per culture flask for different durations of growth of *Pythium sylvaticum* .

Digestion with restriction enzymes

Approximately 1 µg of DNA was digested with 10 units of EcoRI, HindIII, or BamHI. Digests were incubated at 37°C for 2 h, heated at 65°C for 10 min to stop reaction and put on ice before loading onto gels. Enzymes with their respective buffers were obtained from BRL.

Gel electrophoresis

The DNA aliquots were mixed with water and concentrated loading buffer to a final concentration of 1.5% Ficoll 400 and 0.025% bromophenol blue. Electrophoresis was done overnight in 0.7-0.8% agarose gel with 1 µg ethidium bromide/ml, in a TBE buffer (89 mM tris base, 89 mM boric acid, and 2.5 mM Na_2EDTA) and at 0.7 V/cm (Davis *et al.* 1980). A 1 kb DNA ladder (BRL) was used as a size marker. The gels were photographed over a 300 nm UV light source for recording band patterns.

Transfer to nylon membrane

The gel was put in 0.25 M HCl for 20 min with a change of solution after 10 min. The solution was sucked out and the gel was rinsed with double distilled water. The water was then replaced by a 1.5 M NaCl, 0.5 M NaOH

solution for 30 min, with a change of solution after 15 min. This was replaced by a 1 M NH_4OAc, 0.02 M NaOH solution for 1 h, with a change of solution after 30 min. One directional transfer onto a Gene Bind nylon membrane (Pharmacia) was done following the technique by Smith and Summers (1980). Filters were baked at 80°C under 760 mm Hg vacuum for 1 h.

Preparation of DNA probes

DNA from an isolate of *P. sylvaticum* Campbell and Hendrix was digested with EcoRI and random fragments were ligated (T4 DNA ligase, BRL) into EcoRI digested PVZ1 plasmid vector provided by Dr. Henikoff (Henikoff and Eghtedarzadeh 1987). The plasmids were then transformed into competent *Escherichia coli* cells, strain JM83, and plated on Luria-Bertani (LB) agar containing 40 µg/ml of 5-bromo-4-chloro-3-indonyl-D-galactopyranoside, 160 µg/ml isopropyl-d-thiogalactopyranoside and 100 µg/ml ampicillin (Snutch 1984). The white colonies, which contained *Pythium* fragments, were transferred and grown overnight in a LB liquid medium. Plasmids were extracted following an alkaline lysis miniprep procedure (Maniatis *et al.* 1982). Plasmids were digested with EcoRI, electrophoresed on a low melting agarose gel, and *Pythium* DNA fragments were extracted and purified (Ogden and Adams 1987). Bacterial colonies containing either a 7kb ribosomal (pCes370, Ellis *et al.* 1986), 1kb 5S (Nelson and Honda 1985), 6kb heat shock (*hsp-1,* Snutch *et al.* 1988), or 3kb actin (*act-1,* Krause *et al.* 1989) probes from *Caenorhabditis elegans* (Maupas) Dougherty were subcultured and the probes were extracted and purified following the same method as above. Approximately 50 ng of each probe was radiolabelled with ^{32}-P dCTP

(Amersham) using an oligolabelling kit (Pharmacia). The loose nucleotides were separated from the probe by a Sephadex spun column (Maniatis 1982). The labelled probes were diluted in a hybridization solution containing 5xSSPE (1x SSPE= 0.18 M NaCl, 10mM sodium phosphate, 1 mM EDTA, pH 7.4), 2.5xDenhardt (1x Denhardt =0.02% Ficoll, polyvinylpyrrolidone and BSA) and 0.3% sodium dodecyl sulphate (SDS) to reach a final concentration of $5x10^6$ Cerenkov cpm (Davis *et al.* 1980). The radioactive solution was transferred to a polypropylene tube after being passed through a 0.2-µm millipore filter attached to a 30 ml-disposable syringe, and was then placed in boiling water for 15 min.

Hybridization

Filters were prehybridized at 65°C for a minimum of 1 h in 5xSSPE, 2.5xDenhardts, 0.3%SDS solution. This solution was poured out and hybridization solution with radiolabelled probe was added for a total volume of 10 ml /100cm^2 of membrane. Filters were incubated at 65°C for 18 h and washed at least six times in 0.2xSSPE, 0.2% sodium dodecyl sulphate at 42°C for a minimum total time of 1.5 h. Filters were air dried, autoradiographed (Kodak X-Omatic K) at -70°C using an intensifying screen (Dupont lightning +) and the film was developed. The patterns of bands were observed and recorded.

Host specificity and pathogenicity

The following technique is a modified version of the one described by Chamswarng and Cook (1985). Four isolates were used, two *P. sylvaticum* isolates, one isolated from wheat (WM2c) and one from beans (BM2a), and two

P. ultimum isolates, one from wheat (WM2b) and one from beans (BM4b). Each isolate was part of a different cluster. The mycelium and V-8 broth culture medium were placed on a #1 Whatman filter paper in a 150-mm Buchner funnel and the liquid was removed by suction. Mycelium was rinsed with at least 0.5 L of sterile distilled water, and free water was drawn out by suction. A mycelial suspension was made by blending 3.0±0.1 g of mycelium with 200 ml of 0.1% water agar at medium to high speed for 20 sec in a Sorval homogenizer. The suspension was added to 4 kg of autoclaved mineral soil. Three days later, the same amount of mycelium was added in 100 ml of 0.1% water agar. Control soil was inoculated with 0.1% water agar. In aseptic conditions, the soil was mixed with the inoculum in plastic trays (30x35x10 cm) sterilized with 95% ethanol. Final matric potential was -0.16 bar. Trays were covered with aluminum foil, and left in the dark at 25°C, 95% relative humidity. The soil was mixed 8 days after the first inoculation and again 6 days later. Populations of *Pythium* spp. were estimated immediately after the second mixing by dilution plating with 2 ml of aliquots (20 g of soil in 100 ml of water, one composite sample/tray with triplicate plates) on *Pythium* selective medium (Mircetich 1971). CFU's were estimated 24 hours later. The populations in the trays were then immediately adjusted to 260 CFU/g by mixing an appropriate amount of sterile soil and 370 g portions were put in ten 12x12cm trays. Seeds were surface sterilized in agitated 1% NaOCl for 2 min and rinsed with sterile distilled water. Nine bean seeds were planted in a 3x3 grid pattern in each of five trays and nine wheat seeds in each of five other trays. Seeds were planted 1 cm deep with sterilized forceps and holes were covered using the forceps. The amount of dry soil in each pot was known and matric potential was adjusted daily to -0.1 bar with

sterile distilled water, as described in the previous chapter. Plants were grown at 25°C, 95% relative humidity, under a light intensity of 250 µEin m^{-2} s^{-1} and a 16:8 light:dark regime. Two weeks after planting emergence as well as the dry weight of roots and shoots was assessed for each pot. The dry weight values per pot were divided by the number of plants emerged before analysis. Surface sterilized ungerminated seeds, crown and root segments were plated on Mircetich selective medium (1971) for reisolation of *Pythium* spp.

Statistical analysis

For the DNA variability, a separate analysis was made for the eight and 53 isolates collections. For each isolate, the data were recorded as a set of zeros and ones corresponding to the absence or presence of particular bands. A matrix Z of zeros and ones was generated with fungal isolates as rows and bands of interest as columns. A new matrix was generated by multiplying the Z matrix by its transpose Z' giving a new matrix M=Z Z' with fungal isolates as rows and columns. The numbers in the diagonal of the matrix M represent the total number of bands in a particular isolate (n_{xx} or n_{yy}) and the other numbers represent the number of bands shared by two particular isolates (n_{xy}). A Fortran program was designed to calculate dissimilarity coefficients for all pair comparisons using the matrix M and the following formula:

$$D = 1 - \left[\frac{2n_{xy}}{n_{xx} + n_{yy}}\right].$$

From these values, a cluster analysis by the unweighted pair-group method with average (UPGMA) was performed and a tree was constructed (Denny *et al.*

1988, SAS 1985). Fisher exact test was performed with three pairs of main clusters and binomial hypothesis tests were done for smaller clusters within the main clusters to find if there was any evidence of host specificity within fungal species. Emergence data for the pathogenicity and host specificity experiment were analyzed using GLIM and logistic regression (see Chapter 1).

RESULTS

Optimal growth time

The optimal time to grow *P. sylvaticum* in V-8 broth liquid culture at 26°C was 8 to 11 days (Fig. 11). There was no noticeable shearing of DNA when uncut DNA was electrophoresed for all the incubation periods investigated. Therefore, an incubation time of 9 to 10 days was selected.

Eight isolate model

Identification from Ottawa

There was no evidence of host specialization at the species levels in the eight representative isolates sent for identification to Ottawa. Four out of the eight isolates were identified as *Pythium sylvaticum* and were described as heterothallic and the other four were identified as *Pythium ultimum* Trow (Table 6). They were divided equally between bean and wheat hosts. Both species were found as colonizers in the two different soil types.

Table 6. Origin, identity[a] and code name assigned to representative *Pythium* spp. isolates obtained from the roots of glyphosate treated wheat and bean seedlings[b].

HOST	*Pythium* spp. AND CODE	
	MINERAL SOIL (M)	ORGANIC SOIL (O)
Beans (B)	*P. sylvaticum* BMI	*P. sylvaticum* BOI
	P. ultimum BMII	*P. ultimum* BOII
Wheat (W)	*P. ultimum* WMI	*P. sylvaticum* WOI
	P. ultimum WMII	*P. sylvaticum* WOII

[a] Identifications made by Dr. D.J.S. Barr, Biosystematics Research Institute, Agriculture Canada, Ottawa.

[b] Two isolates (I and II) obtained from two different seedlings for each plant species-soil type combination.

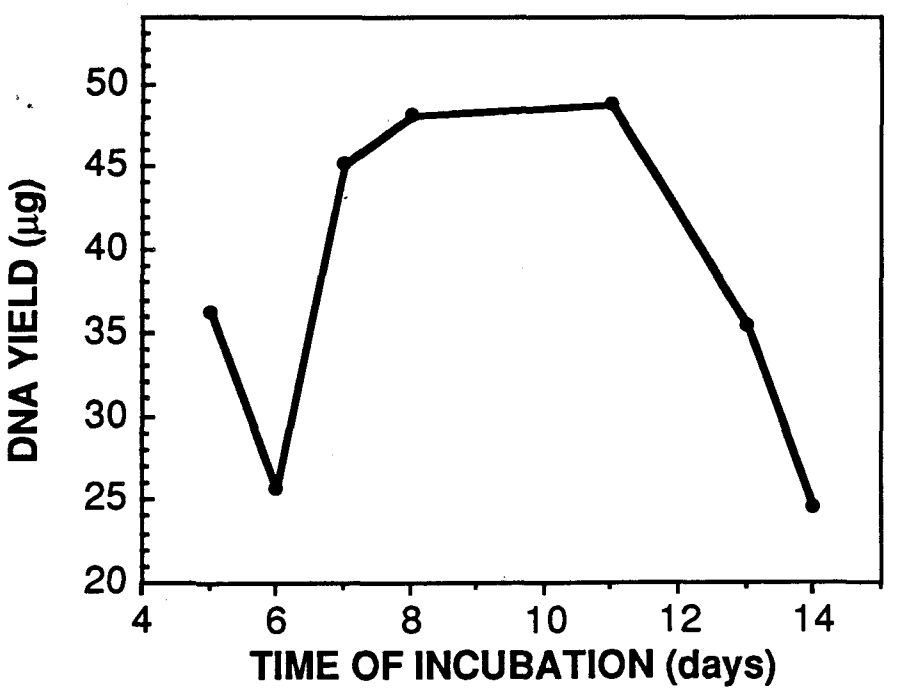


Figure 11. Total yield of DNA from different cultures of an isolate of *Pythium sylvaticum* grown in 50 ml of V-8 broth as a function of time of incubation of the culture at 26°C.

Restriction digests

After digestion of the DNA with either HindIII or EcoRI, five different bands for each restriction enzyme differentiating between some isolates of the same species could be observed (Fig. 12). The size of any diagnostic band of repeated DNA sequences varied from 2.5 to 6.0 kb.

Autoradiogrammes

Of all the four heterologous probes from *C. elegans*, only the ribosomal probe could differentiate within *Pythium* spp. when hybridized to size fractionated BamHI digest (Fig. 13). Ribosomal probe hybridized to DNA from HindIII digest shows potential for species identification but was not used for the cluster analysis. The 2.5 kb random probe #2 from *P. sylvaticum* showed some differences within the *P. sylvaticum* isolates for both HindIII and EcoRI digests (Fig. 14).

Cluster analysis

From the matrix of zeros and ones obtained from band observations, the dissimilarity coefficients were calculated and are shown in Table 7. The first obvious grouping from the analysis as shown by the dendrogramme (Fig. 15) separates the two different species, *P. ultimum* at the top and *P. sylvaticum* at the bottom. The second division separates the *P. sylvaticum* isolates obtained from wheat from the ones obtained from bean seedlings. In *P. ultimum*, the first separation differentiates between isolates from organic and mineral soils.

Figure 12. Differentiation of *Pythium sylvaticum* and *P. ultimum* isolates by detection of restriction fragment length differences of repetitive DNA in total genomic DNA after digestion with either HindIII or EcoRI enzymes followed by electrophoresis. Two isolates (I-II) were obtained from wheat (W) and from bean (B) hosts grown in mineral (M) and organic (O) soils. A 1 kb ladder (BRL) is shown as a size marker on each side.

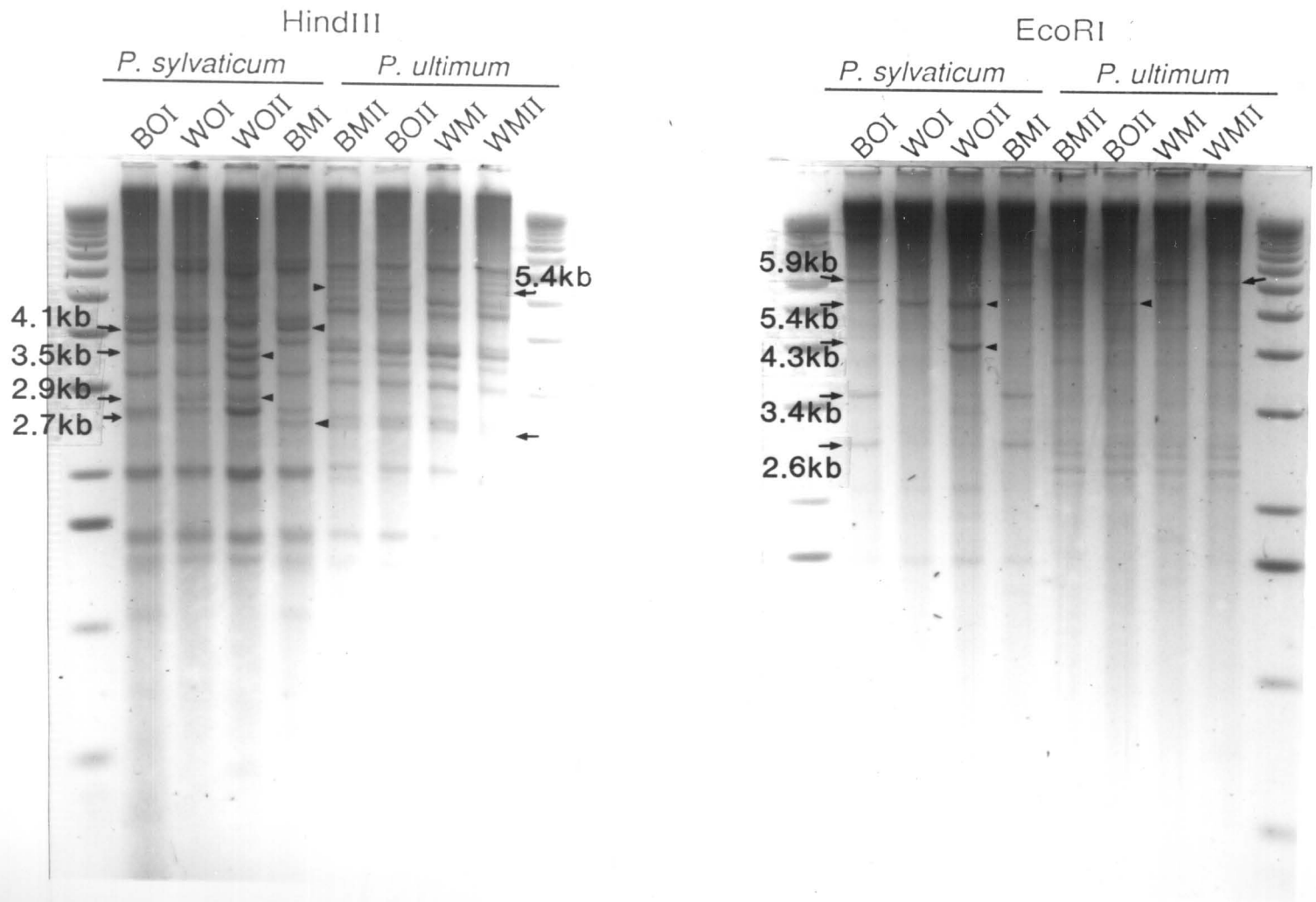

HindIII
P. sylvaticum
P. ultimum
BOI
WOI
WOII
BMI
BMII
BOII
WMI
WMII
4.1kb
3.5kb
2.9kb
2.7kb
5.4kb
EcoRI
P. sylvaticum
P. ultimum
BOI
WOI
WOII
BMI
BMII
BOII
WMI
WMII
5.9kb
5.4kb
4.3kb
3.4kb
2.6kb

Figure 13. Autoradiogramme of a Southern blot of electrophoresed digests (EcoRI, HindIII, BamHI) of total DNA from *Pythium sylvaticum* and *P. ultimum* after hybridization under low stringency with a ribosomal probe from *Caenorhabditis elegans*. Two isolates (I-II) were obtained from wheat (W) and from bean (B) hosts grown in mineral (M) and organic (O) soils.

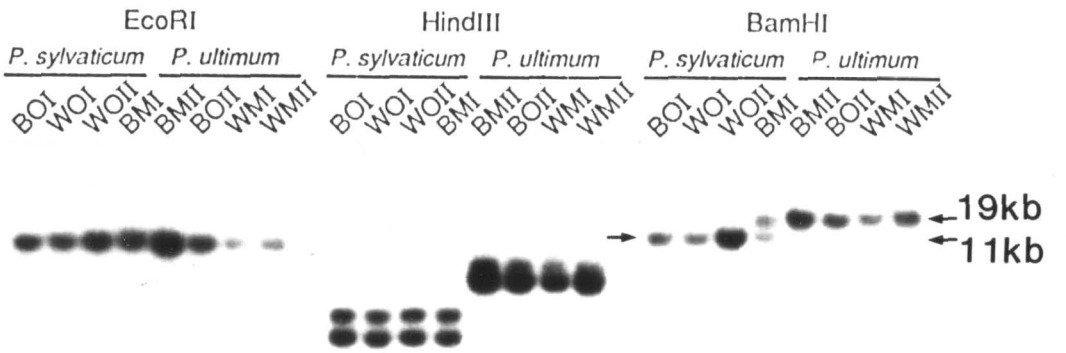

EcoRI
HindIII
BamHI
P. sylvaticum
P. ultimum
BOI
WOI
WOII
BMI
BMII
BOII
WMI
WMII
19kb
11kb

Figure 14. Autoradiogramme of Southern blots of electrophoresed digests [HindIII (left), EcoRI (right)] of total DNA from *Pythium sylvaticum* and *P. ultimum* after hybridization under low stringency with a 2.5 kb anonymous *P. sylvaticum* probe shown on the right blot. The dark bands in the outside lane of each gel are size markers. Two isolates (I-II) were obtained from wheat (W) and from bean (B) hosts grown in mineral (M) and organic (O) soils.

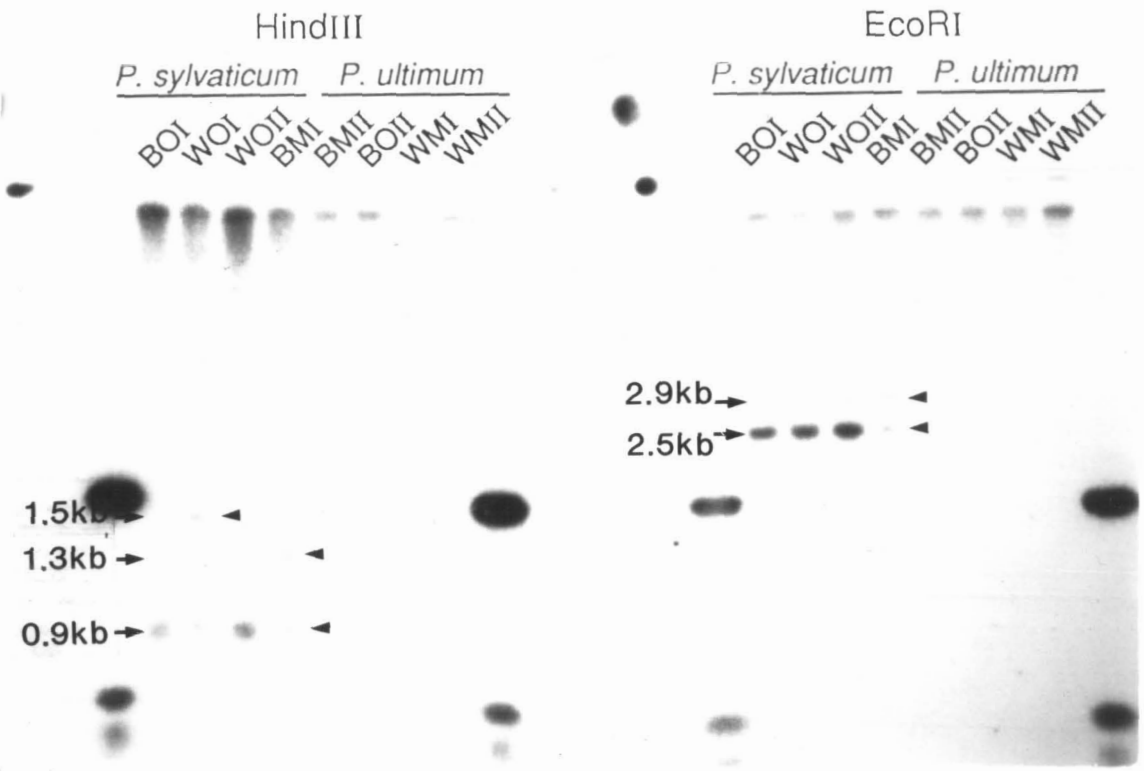

HindIII
P. sylvaticum
P. ultimum
BOI
WOI
WOII
BMI
BMII
BOII
WMI
WMII
1.5kb
1.3kb
0.9kb
EcoRI
P. sylvaticum
P. ultimum
BOI
WOI
WOII
BMI
BMII
BOII
WMI
WMII
2.9kb
2.5kb

Table 7. Dissimilarity coefficients for the different pairs of *Pythium* spp. isolates obtained from bean (B) or wheat (W) hosts grown in either mineral (M) or organic (O) soils. The first four isolates are *P. sylvaticum* and the next four are *P. ultimum.*

	BOI	WOI	WOII	BMI	BMII	BOII	WMI	WMII
BOI	0.000	.	.	.	.	.	.	.
WOI	0.467	0.000	.	.	.	.	.	.
WOII	0.600	0.286	0.000	.	.	.	.	.
BMI	0.158	0.556	0.667	0.000	.	.	.	.
BMII	0.600	1.000	0.714	0.556	0.000	.	.	.
BOII	0.733	0.857	0.571	0.667	0.143	0.000	.	.
WMI	0.571	1.000	0.692	0.529	0.077	0.231	0.000	.
WMII	0.600	1.000	0.714	0.556	0.000	0.143	0.077	0.000

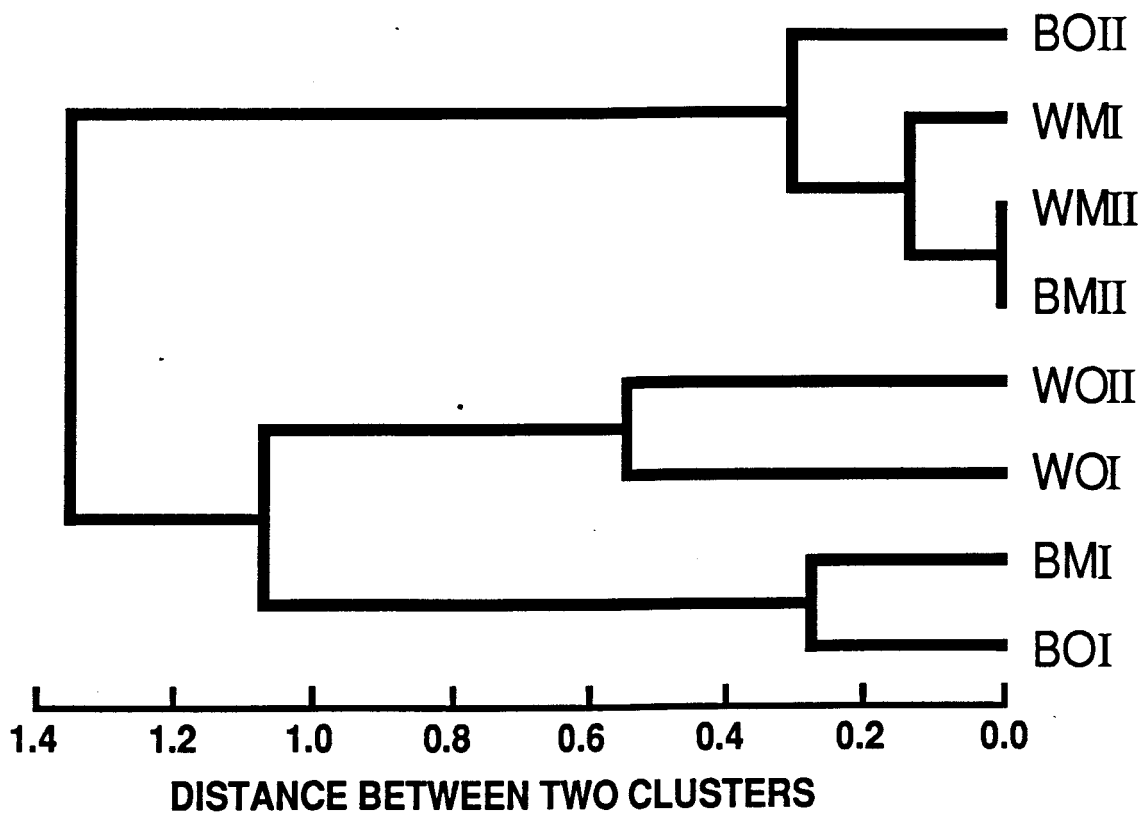


Figure 15. Dendrogramme derived from cluster analysis using 10 characteristic bands of repeated DNA from HindIII and EcoRI restriction digests and seven bands from restriction fragment length polymorphism using an anonymous probe from *Pythium sylvaticum* and a heterologous probe from *Caenorhabditis elegans.* Two *Pythium* spp. isolates (I and II) came from different seedlings of either beans (B) or wheat (W) grown in either mineral (M) or organic (O) soil. The four isolates at the top were identified as *P. ultimum* and the four at the bottom as *P. sylvaticum.*

Fifty-three isolate model

The root systems of wheat or bean seedlings were heavily colonized by *Pythium* spp. 2 days after glyphosate treatment (Table 8). Colonization by *Fusarium* spp. was below 2 CFU per plant.

Using the same bands selected for the eight isolate model, cluster analysis was performed and the resulting dendrogramme is shown in Fig. 16. Of the total number of isolates, 23 came from wheat and 30 from beans. The first main differentiation separated the *P. ultimum* (cluster A) from the *P. sylvaticum* (cluster B) isolates. Within *P. ultimum*, 10 isolates came from wheat and 18 from beans and within *P. sylvaticum* , 9 came from wheat and 11 from beans (Table 9). These bean:wheat ratios within each *Pythium* spp. are not significantly different from the overall ratio of bean and wheat hosts. Therefore, there was no tendency to isolate more of a given *Pythium* spp. from one host than from another. There were no evidences of host or site specificity in any of the clusters differentiating within species. With respect to site, 23 isolates came from the organic soil and 30 from the mineral soil. As shown in Table 9, most of the *P. sylvaticum* were found in the mineral soil and a majority of *P. ultimum* were found in organic soil. The *Pythium* colonists differed between the two sites.

Table 8. Average colony forming units per root system plated on Mircetich (1971) or Nash and Snyder (1962) selective media based on a total of five plants for each treatment combination. Wheat and bean seedlings were 12 days old at the time of glyphosate treatment, received 2.5 μg and 10μg of glyphosate, respectively, and were plated 2 days after glyphosate treatment.

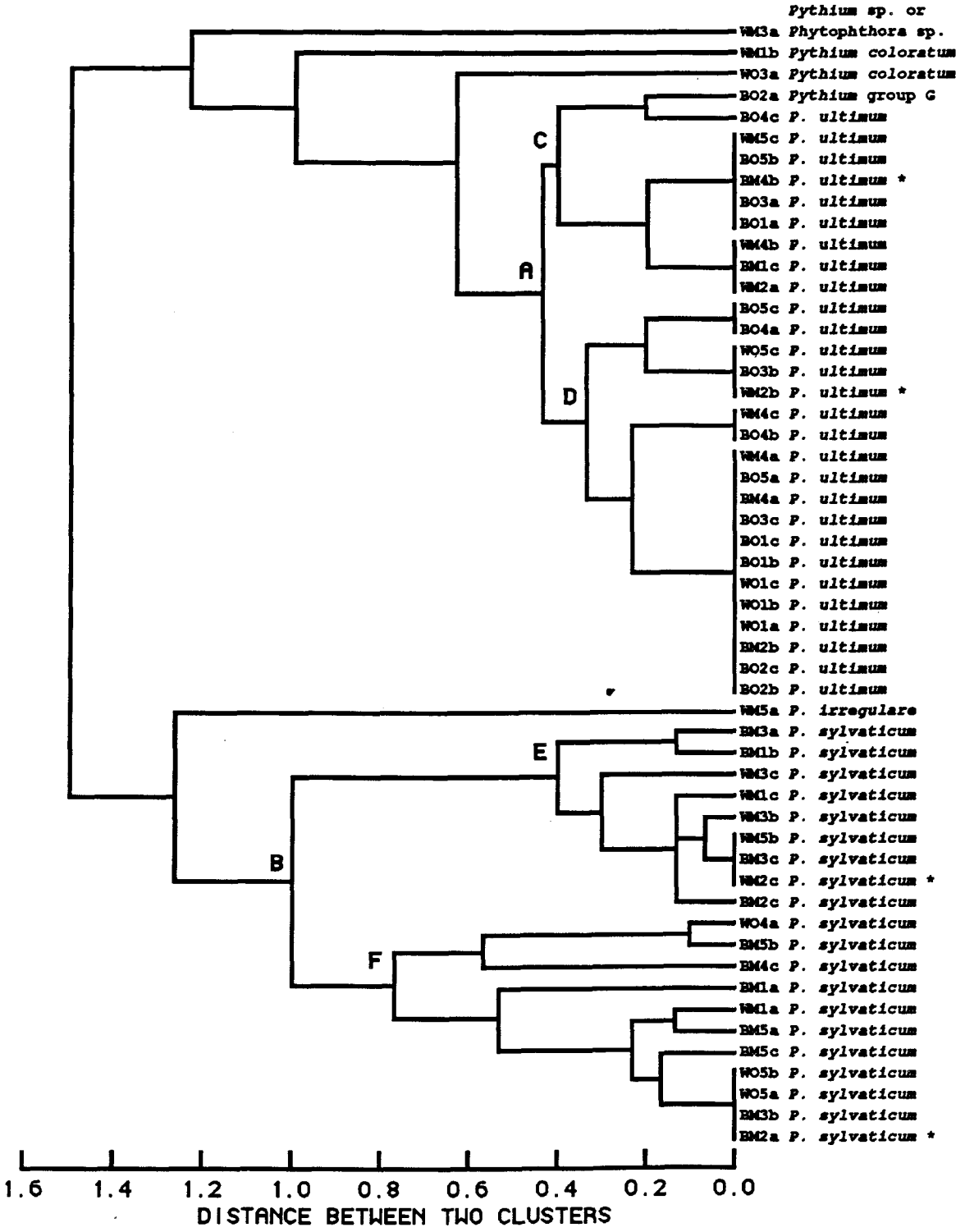
		Pythium spp. CFU		*Fusarium* spp. CFU	
HOST	SOIL	CONTROL	GLYPHOSATE	CONTROL	GLYPHOSATE
Wheat	Mineral	0.3	13.8	0.0	1.0
Wheat	Organic	0.4	3.4	0.0	0.2
Beans	Mineral	0.0	13.2	0.0	0.4
Beans	Organic	0.5	10.0	0.0	1.4

Table 9. Number of isolates of *Pythium sylvaticum* and *P. ultimum* found in different clusters with regard to possible host or site specificity in the two fungal species or in some of the groupings within species. The values are arranged in 2x2 contingency tables that refer to the clusters shown in Fig. 16.

		NUMBER OF ISOLATES PER CLUSTER			
		HOST SPECIES		SOIL TYPE	
Pythium sp.	CLUSTER	WHEAT	BEANS	MINERAL	ORGANIC
P. ultimum	A	10	18	10	18 *
P. sylvaticum	B	9	11	17	3
P. ultimum	C	3	6	5	4
P. ultimum	D	7	12	5	14
P. sylvaticum	E	5	4	9	0
P. sylvaticum	F	4	7	8	3

* Significant difference at the 0.05 level estimated by Fisher exact test.

Figure 16. Dendrogramme derived from cluster analysis using 10 characteristic bands of repeated DNA from HindIII and EcoRI restriction digests and seven bands from restriction fragment length polymorphism using an anonymous probe from *Pythium sylvaticum* and a heterologous probe from *Caenorhabditis elegans*. There was a total of 53 different *Pythium* spp. isolates. Up to three isolates (a-c) came the roots of 5 (1-5) different wheat (W) or bean (B) glyphosate treated seedlings, grown in either mineral (M) or organic (O) soil. Clusters **C-F** represent within species grouping described in Table 3. Isolates followed by * were used for *in vivo* pathogenicity and host specificity experiment. Representative isolates of *P. sylvaticum* and *P. ultimum* and all other species were identified by Dr. D.J.S. Barr of the Biosystematics Research Center, Agriculture Canada, Ottawa.


Pythium sp. or
WM3a Phytophthora sp.
WM1b Pythium coloratum
WO3a Pythium coloratum
BO2a Pythium group G
BO4c P. ultimum
WM5c P. ultimum
BO5b P. ultimum
BM4b P. ultimum *
BO3a P. ultimum
BO1a P. ultimum
WM4b P. ultimum
BM1c P. ultimum
WM2a P. ultimum
BO5c P. ultimum
BO4a P. ultimum
WO5c P. ultimum
BO3b P. ultimum
WM2b P. ultimum *
WM4c P. ultimum
BO4b P. ultimum
WM4a P. ultimum
BO5a P. ultimum
BM4a P. ultimum
BO3c P. ultimum
BO1c P. ultimum
BO1b P. ultimum
WO1c P. ultimum
WO1b P. ultimum
WO1a P. ultimum
BM2b P. ultimum
BO2c P. ultimum
BO2b P. ultimum
WM5a P. irregulare
BM3a P. sylvaticum
BM1b P. sylvaticum
WM3c P. sylvaticum
WM1c P. sylvaticum
WM3b P. sylvaticum
WM5b P. sylvaticum
BM3c P. sylvaticum
WM2c P. sylvaticum *
BM2c P. sylvaticum
WO4a P. sylvaticum
BM5b P. sylvaticum
BM4c P. sylvaticum
BM1a P. sylvaticum
WM1a P. sylvaticum
BM5a P. sylvaticum
BM5c P. sylvaticum
WO5b P. sylvaticum
WO5a P. sylvaticum
BM3b P. sylvaticum
BM2a P. sylvaticum *
A
B
C
D
E
F
1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
DISTANCE BETWEEN TWO CLUSTERS

Pathogenicity tests

Plant emergence

Two weeks after seeding, both *Pythium* spp. had reduced the emergence of wheat seedlings by at least 75% (Table 10). In beans, only *P. ultimum* caused a significant reduction in emergence. There was no indication of host specialization among the isolates tested, in the sense of greater pathogenicity toward seeds of a plant species by *Pythium* isolates obtained from that plant species compared with those obtained from the other plant species.

Recovery of *Pythium* spp.

The *Pythium* spp. corresponding to the inoculum used were recovered from surface sterilized ungerminated seeds, crown and roots of bean and wheat.

Table 10. Percent emergence based on five replicates with nine seeds each for different treatments. Two *Pythium* spp. were tested and isolates came from glyphosate treated wheat and bean hosts. Autoclaved mineral soil was inoculated with a hyphal suspension in 0.1% water agar. Control soil received only the 0.1% water agar.

SOIL INOCULUM		% EMERGENCE	
Pythium sp.	HOST	WHEAT	BEANS
P. sylvaticum	Wheat	11.1 *	95.6
P. sylvaticum	Beans	13.3 *	88.9
P. ultimum	Wheat	8.9 *	40.0 *
P. ultimum	Beans	0.0 *	42.2 *
Control soil	N/A	62.2	100
Germination on moist filter paper	N/A	71.1	97.7

* Significant difference ($p<0.05$) from control soil tested with Scheffé's multiple contrasts.

Root and shoot dry weight

The dry weights of shoots or roots per emerged 2-week old seedling were not significantly affected by the soil inoculation with any of the fungal isolates.

DISCUSSION

When eight isolates were used, the grouping within *P. sylvaticum* correlated with the host, but the groupings derived from the DNA characterization of 53 isolates did not correlate with the hosts from which these isolates were obtained, illustrating the risks associated with testing hypotheses with small sample size. However, *P. ultimum* was more prevalent in glyphosate treated plants grown in organic soil and *P. sylvaticum* was found more in glyphosate treated plants grown in mineral soil in the 53 isolates sample.

The *Pythium* colonists of glyphosate treated plants can be pathogenic on plants not predisposed by glyphosate. They are not exclusively saprophytic organisms colonizing the roots of dying plants. The majority of *Pythium* spp. show some degree of pathogenicity (Van der Plaats-Niterink 1981), therefore, it is not a total surprise to find that the *Pythium* colonists of glyphosate treated plants are pathogenic. All the ten *Pythium* spp. or varieties isolated from the roots of wheat grown in eastern Washington or northern Idaho were pathogenic to various levels (Chamswarng and Cook 1985).

The *Pythium* spp. colonizing glyphosate treated plants differed between the two soils tested. There might be some potential for using glyphosate treated

plants as a bioassay for determining the disease potential in a given soil. Some *Pythium* spp. are more pathogenic than others and colonizers of glyphosate treated plants may represent the species most likely to cause problems to the crop. Such technique would be faster that dilution plating and faster than *in vivo* tests. This potential use was probably exemplified in my LD_{50} experiment with apple seedlings grown in orchard and virgin soil. No differential sensitivity to glyphosate between plants grown in heat treated and raw virgin soil was found whereas a significant difference was detected in an orchard soil. A mixture of the herbicides paraquat and diquat has been used to detect leaf infection more rapidly. Early infection of lupins (*Lupinus angustifolius* L.) by *Phomopsis leptostromiformis* (Kuhn) Babek is difficult to detect and can be made more conspicuous by treating test plants with paraquat (Cowling *et al.* 1984). Tools for research in epidemiology, ecology or disease resistance could be designed with herbicide treated plants. Based on the results I have obtained concerning host specialization of colonizers of glyphosate treated plants, it is unlikely that highly specialized colonizers would be preferentially detected with such a technique. Canaday *et al.* (1986) reported that charcoal rot of soybean caused by *Macrophomina phaseolina* (Tassi) Goid was not increased by applying sublethal doses of glyphosate (0.28 kg/ha). Generally, highly specialized parasites can attack hosts that are not weakened and in fact prefer healthy hosts. These specialized parasites may also react slowly and be outcompeted by facultative parasites in the 'race' to colonize a plant stressed by glyphosate.

Curran *et al.* (1986) differentiated between races of *Meloidogyne arenaria* (Neal) Chitwood, *M. hapla* Chitwood and *M. incognita* using characteristic bands of repetitive DNA. I was able to find several differences

within *P. ultimum* and *P. sylvaticum* using a similar technique. The use of DNA probes is considered to be a more sensitive technique than the use of repetitive DNA bands. My results with DNA probes showed differences within *P. sylvaticum* but not within *P. ultimum.* I made probes only from *P. sylvaticum,* therefore my chances to find differences within *P. sylvaticum* were likely higher than within *P. ultimum* since the probes tested did not even hybridized with *P. ultimum.* The other reason for selecting *P. sylvaticum* was because it is a heterothallic species (Campbell and Hendrix 1967). It reduces the risk of variation during the subculturing process of a particular isolate. *P. ultimum* is generally a homothallic species and could potentially undergo sexual recombination during subculturing process. If this phenomenon occurred, its effects were undetected since no differences in banding pattern of electrophoresed restriction digests of any of the original four *P. ultimum* isolates were found among DNA preparations of the same isolates made at various times.

The numerous bands from electrophoresed restriction digest of total DNA have great potential for being diagnostic for species identification (Fig. 12). *Pythium* DNA can be separated by CsCl density gradient centrifugation into different bands that are located at distinct places on the gradient depending on the species (Belkhiri and Dick 1988). Mitochondrial DNA (McNabb *et al.* 1987) and ribosomal DNA (Klassen *et al.* 1987) also vary among *Pythium* spp. The analysis of restriction fragment length differences of repeated DNA sequences for total DNA as performed here has the main advantage of being done without high speed centrifugation or radioisotopes used for the techniques described above. Repetitive DNA polymorphism analysis was used successfully for

differentiating *Phytophthora* species, a genus closely related to *Pythium* (Panabières *et al.* 1989). DNA extraction, digestion and electrophoresis are less time consuming than repetitive measurement of reproductive structures and also have the advantage of making the production of sexual structures unnecessary. However, it is not presently a primary basis for species identification so there would have to be standards for comparison, derived from accepted taxonomic approaches. An extensive amount of work would have to be done to find characteristic bands and to verify that these bands are, in fact, diagnostic for many different isolates of the same species. However, given these caveats, identifying *Pythium* spp. would become easier and faster than with the taxonomic techniques normally used. Quite possibly, the results of using these techniques may lead to revision of present taxonomic classification within this group of fungi.

CONCLUSION

Glyphosate is one of the best-selling herbicides worldwide and it is unlikely that its popularity will decrease in the near future. Stauffer Chemical, who tried to market Touchdown, a product very similar to glyphosate, was brought to court by Monsanto and lost. Now ICI is the new owner of the agricultural chemicals unit of Stauffer and has reached agreement with Monsanto to sell Touchdown in the USA in 1990 when the patent for glyphosate expires (Storck 1988). Monsanto will provide the *N* -phosphonomethylglycine intermediate for the production of Touchdown and ICI will have the uncontested license to sell Touchdown in some selected countries where the patent on glyphosate is still in effect. In spite of such a deal, it is anticipated that the price of glyphosate will decrease substantially as soon as the patent expires in North America. There are predictions of a new price of $6 per liter (Wilkins 1988), a four-fold drop. The amount of glyphosate used for the practices currently registered will certainly increase and because of the decrease in price, new utilizations that are presently not economical will be possible. Some analysts predict sales, in western Canada alone, of $240 to $360 millions per year (Wilkins 1988). For example, growing crops with minimum tillage is already common practice and, with the irreversible trend toward sustainable agriculture, it may soon be the only acceptable way to grow the majority of field crops. This fact combined with a decrease in price of glyphosate will certainly increase its use. Glyphosate has dominated the herbicide market for several years and, 5 years from now, the present period might be considered as the end of the lag phase before exponential growth.

Importance of fungal colonizers

Questions about the role of fungal colonizers in the herbicidal action of glyphosate are of academic and practical significance. Field studies about the role of fungal colonizers in the herbicidal action of glyphosate are virtually non-existent, and field studies on the indirect effect of the colonizing fungi on crops are rare. This discussion is mostly speculation but I believe that it should be done. First, I suspect that differences in soil microflora and interactions with the soil environment are responsible for some of the variations in the efficacy of glyphosate observed in the field. Second, it is known that rapid fungal colonization of weeds occurs after glyphosate treatment (Lévesque *et al.* 1987) and I think that the damage caused by the buildup of the fungal colonizers often goes unnoticed or is blamed on fungi without suspecting that the herbicide treatment is the cause. Third, non target plants often receive sublethal doses of glyphosate and I believe that disease levels in these plants can be substantially increased. I will discuss these three points in more detail.

Johal and Rahe (1984) and I have shown that for a given plant species and dose of glyphosate, microorganisms are required to kill the plants and that it takes more glyphosate to kill plants in the absence of certain microorganisms. There is an immediate need to standardize the methods of investigations with respect to microorganisms and herbicidal efficacy. The strict minimum would be to mention what was used as potting soil and if it were pasteurized, fumigated or sterilized. It becomes technically more difficult to verify the role of the microorganisms in the field but I strongly believe that in certain cases the

efficacy of glyphosate varies because of differences in microflora. I also suspect that for some plant species that have a small leaf surface or in plants that have received a low amount of herbicide because of non uniform spraying, the microorganisms are significant synergists to the herbicidal action. Variations in field efficacy in fairly similar environmental conditions are substantial (Caseley and Coupland 1985), probably too substantial to be caused only by differences in translocation or absorption.

I have demonstrated that glyphosate treated plants are readily colonized by fungi, primarily *Pythium* and *Fusarium* spp., almost immediately after herbicide application. In the present study the *Pythium* colonizers were pathogenic and not specialized for either wheat or beans. Lévesque *et al.* (1987) showed in the field that *Fusarium* spp. quickly colonize weeds treated with recommended doses of glyphosate and that the number of propagules in the weed roots or in the soil increased after glyphosate treatment of the weed cover. They did not observe a detrimental effect on the subsequently planted crops, but such increases may be detrimental to the crop (Lynch and Penn 1980, Mielke 1983). When a crop is seeded immediately after glyphosate treatment of a thick weed cover, the new roots grow in a soil that has a high density of small thalli of pathogenic fungi attached to the roots of the weeds. The propagule density and inoculum potential of the pathogenic fungi would be increased and the vigor of the mycelium would be greater because of the nutrient base provided by the weed roots. In British Columbia, it is not uncommon to treat apple trees with glyphosate before removing them and establishing a new orchard. Such practice should be stopped until more is learned about the potential long term problems with respect to root diseases.

There are only a few controlled field experiments with annual plants to verify if this phenomenon can have detrimental effects on crops and results are conflicting (Lynch and Penn 1980; Mielke 1983; Lévesque *et al.* 1987).

Killing the weed cover with herbicide does not necessarily result in an increase in fungal inoculum. A mixture of diquat, paraquat and dicamba applied to the weed cover was often associated with a decrease in propagule and disease level of *Gaeumannomyces graminis* var.*tritici* (Sivasithamparam and Bolland 1985). Similarly, Barbetti (1984) found that paraquat treatment of weed cover decreased root rot in germinating subterranean clover (*Trifolium subterraneum* L.).

There are several registered uses of glyphosate in which a sublethal amount of of glyphosate can potentially reach the crop. There are three categories of such uses. The first one is directed application using tools or techniques such as a rope wick, or spraying around plants. With either of these techniques, it is unavoidable that small amounts of glyphosate reach some of the crop plants. An example is apple trees receiving glyphosate on their bark. Our research programme on glyphosate was started after some empirical observations on young apple trees that accidentally received a drop or two of diluted Roundup on their bark. These trees were tagged and the next year showed poor vigor which appeared to be associated with root rot. If the use of glyphosate is to increase in orchards, it is essential to know that it will not increase the incidence of root rot and cause yield losses larger than what would have been caused by the weeds. The second way sublethal doses of glyphosate can reach the crop is by spraying when absorption or translocation

in the crop is minimal compared to the target weeds. This is how glyphosate is applied on conifers and it provides rapid increase in growth of desired tree species because of reduced competition (Lund-Høie 1985; Willis *et al.* 1989). There are definitely short term benefits in using glyphosate but it is not known if the trees will suffer more from root rot in the future because of these applications. The third way is to apply low doses that do not seem to damage the crop. Some parasitic plants are controlled this way (Sauerborn 1989; Abu-Irmaileh 1989) and sugarcane, a perennial crop, ripens faster and can be harvested earlier after spraying with glyphosate (Duskey *et al.* 1986). In British Columbia, root rot is very common in our forests and orchards. Is root rot more prevalent in areas where glyphosate has been used? The answer is not known but I think it should be.

New ways of using glyphosate

As mentioned before, the expected drop in price of glyphosate will increase its use for currently registered applications and will open new markets for field crops where its use is presently not economical. Because of reduced price and ongoing research in weed science, horticulture and genetic engineering, there are several new innovative utilizations that should soon be available. For some of these potential new uses, there are longer term problems. For example, when glyphosate was applied to control root suckers on apple trees in the fall, there was yield reduction and noticeable damage the following year (Cowgill and Majek 1988). A similar effect was noticed on raspberry suckers (Freeman 1980). Such delayed effects are difficult to explain as due solely to the direct phytotoxicity of glyphosate.

The most innovative new use for glyphosate will likely come with the introduction of glyphosate tolerant crops. Glyphosate tolerance is one of the most popular model systems in plant molecular biology (Klee *et al.* 1987; Kunze *et al.* 1989). It will probably provide one of the most lucrative genetically engineered products developed from plant molecular biology research. Glyphosate tolerance can be transferred to plants via two basic mechanisms: overproduction of 5-enolpyrulvylskikimate-3-phosphate synthase or mutation of that enzyme which retains enzymatic activity but reduces binding to glyphosate (Kishore *et al.* 1988). Tomato, tobacco and petunia plants tolerant to glyphosate have been engineered (Shah *et al.* 1986; Thompson *et al.* 1987; Kishore *et al.* 1988) and it is presently technically feasible to transfer glyphosate tolerance to any dicotyledonous plant species (Horsch *et al.* 1988). The stability of this tolerance in a field situation is not known yet. Glyphosate tolerant soybean lines were created by Hartwig (1987) using field trials and classical breeding techniques. After a few generations of selection he was able to spray 0.56 kg/ha of glyphosate on the plants without reduction in yield. The following year, in more moist field conditions, this tolerance collapsed and severe damage was observed in the plants designated tolerant. It was concluded that environmental variables were too important in the system and the breeding program was discontinued.

The idea of using glyphosate as a crop desiccant is not new (Whigham and Stoller 1979), but glyphosate is still not registered for this use in Canada. Chemical desiccation can allow earlier harvest which can mean saving the crop in some years. Chemical desiccation also provides preharvest weed control

which facilitates mechanical harvesting, reduces weed seed bank and decreases perennial weed problems. It is expected that registration will be obtained soon and with a decrease in price, there will be a very large amount of glyphosate applied for this particular purpose (Wilkins 1988).

There are ongoing trials to design ways to selectively kill weeds in pasture. The first technique consists in applying glyphosate when absorption and/or translocation is minimal in the desired grazing plant species and is still high in the weed species. This is achieved by either applying glyphosate at a very specific time of the year or after animals have preferentially grazed the desirable plant species (Campbell and Ridings 1988). It was found that plants recently treated with glyphosate are preferentially eaten by cattle (Kisseberth *et al.* 1986) and they suggested that translocation could be minimized and selective control achieved if the animals were left grazing in a pasture that has just been treated.

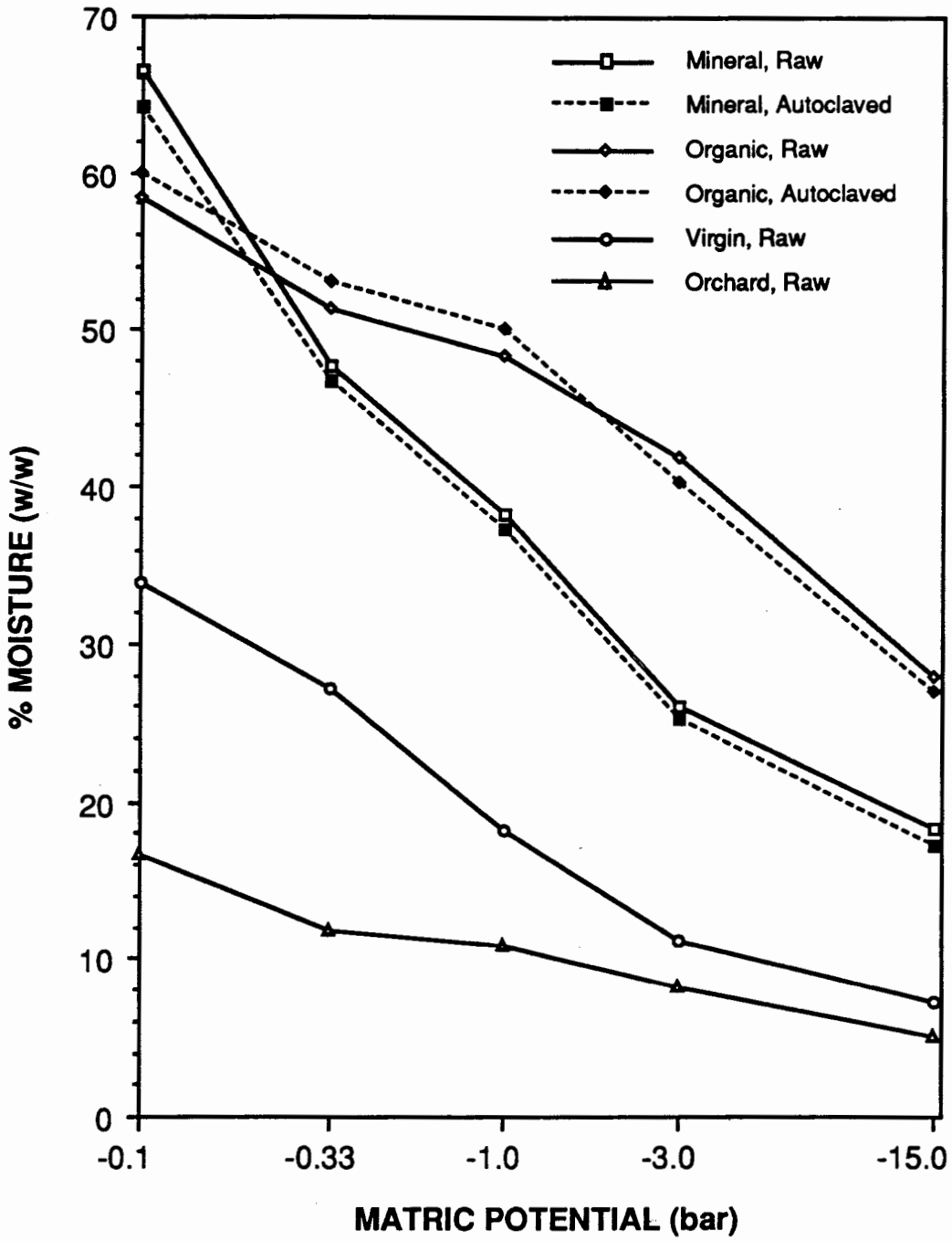
Melanconis alni Tul. is a weak pathogen causing canker on alder, a very important weed in forests of British Columbia. This fungus does not kill the trees by itself. With one twentieth of the recommended dose, glyphosate does not cause any detectable damage to alder but when combined with *M. alni* , the trees die because of the canker (C. E. Dorworth, Pers. Comm., Pacific Forest Center, Forestry Canada, Victoria, British Columbia). This combination of a weak pathogen with a very low dose of herbicide has the advantage of restricting the biocontrol agent to selected geographical zones. This is particularly appropriate when the weed to be controlled is considered a desirable plant in other situations.

Needs for the future

Considering what is known now about the interaction between fungal colonizers and glyphosate treated plants, there are some risks associated with the increased use of glyphosate or with any of the potential new applications. Glyphosate is the most popular herbicide and is likely to become the only herbicide for most of the crops. Some of the problems that already exist should be understood better before the predicted massive increase in the use of glyphosate, otherwise the situation might become difficult to rectify.

An association between increased field use of glyphosate and increased root rot is not yet documented adequately or understood. If root rot is caused by an increase in propagule density after glyphosate treatment, the release of glyphosate tolerant varieties as well as the registration of glyphosate as a desiccant could create a major problem. There is a risk of loading agriculture or forest soil over very large areas with a high level of propagules of pathogenic fungi. Herbicides can increase yields dramatically on a short term basis but decreases might be observed in the long term. Widespread root rot problems would be probably more difficult to solve than the current weed problem. My observations that fungal colonizers of glyphosate treated bean and wheat seedlings are not specialized and are pathogenic make this negative scenario plausible. Other uses such as biocontrol adjuvant, pasture application, sugarcane ripener, weed control in forests could create a problem of disease susceptibility in the crops that have received a low dose of glyphosate.

The success of glyphosate is based on its efficacy in the field and its ability to control some weed species that cannot be controlled by other means. During the early years of glyphosate use it was impossible to anticipate the possible adverse implications I have described. Lydon and Duke (1989) recommended that if disease resistance of crop or weed species is altered by a pesticide, economic thresholds should be revised accordingly. Somerville (1987) recommended that, on a case by case basis, companies should be required to investigate the side effects of pesticides with respect to microorganisms. There are several new herbicides on the market that have the slow acting characteristic of glyphosate (eg. Fusilade, Poast) with which microorganisms could be involved. This needs to be investigated. It is probably impractical to require extensive tests about disease incidence before registration of new herbicides since the cost of the necessary range of tests is already in the double digit millions. However, once a pesticide has been profitable for a long period of time and when another boom in the sale is predicted, any suspicion about higher disease incidence should be investigated thoroughly. It should be demonstrated in long term experiments that glyphosate improves yield more than other weed management techniques regardless of enhanced diseases. Except for a few controlled experiments, the reported cases of higher incidence of disease in the field after the use of glyphosate are only correlations; the effect of glyphosate in these cases still has to be verified. My research has answered some problems and also shows that much more needs to be done.



Water retention curves for two soils of the Fraser Valley, British Columbia, mineral and organic, as well as for two soils from the Okanagan Valley, British Columbia, virgin and orchard. The effect of autoclaving was examined for the two soils from the Fraser Valley.

APPENDIX II

Power Comparisons Between Logistic Regression and Other Methods

Statistical analysis of binomial data causes difficulties in meeting some of the assumptions of analysis of variance (ANOVA) or regression. The arcsine transformation of the proportions (arcsine $\sqrt{\text{proportion}}$) is commonly used to alleviate this problem. Logistic regression can also be used to analyze binomial data. The concept and software for logistic regression were designed in the early seventies (Cox 1970; Lee 1974), and the technique was used in medical research very soon afterward (Minow *et al.* 1977). It is now generally used and recommended in the medical field for analysis of binomial data in epidemiological studies (Lee 1980; Miké and Stanley 1982). The data obtained in the medical field are generally different than the ones obtained in plant pathology in the sense that several parameters are measured on each individual and each one represents a unique experimental unit whereas in plant pathology, the conditions can be made very homogeneous for a large group of plants from which a proportion will be estimated. Therefore, some of the recommendations about using logistic regression in medical research cannot be taken for granted if one wants to extrapolate to plant pathology.

The main concerns in choosing a particular statistical analysis technique are the power and the type I error. The power is the ability to reject the null hypothesis H_0 when H_0 is false. The type I error is committed when a significant difference is detected when in fact H_0 is true. The best analysis technique will

have the highest possible power, *i.e.* it will detect a given difference with a high probability, and will have a type I error corresponding to the significance level selected. With a given statistical technique, one can raise power by increasing sample size. The power is also directly related to the magnitude of the difference between the treatments.

My first objective was to compare the power of logistic regression with the power of some statistical techniques commonly used in plant pathology for analysis of binomial data. The power was estimated for various sample sizes and differences between treatments. So far, there is no mathematical technique that has been found to calculate power of logistic regression, therefore, computer simulations had to be used.

My second objective was to investigate by computer simulation the relationship between sample size and type I error for logistic regression and for other techniques commonly used in analysis of proportions. Schaeffer (1983) reported that maximum likelihood estimates of logistic regression, the equivalent of sums of squares in classical ANOVA or regression, can be biased with small sample sizes thus possibly increasing the probability of committing type I error. He did not specify a minimum sample size to use.

MATERIALS AND METHODS

Data sets were generated using Waterloo Basic programs (Graham *et al.* 1983). The simulated proportions ($\hat{\pi}$) had two components: the fixed sample size (n) and the simulated outcome ($\hat{\mu}$). The random number function of Waterloo Basic generates numbers (λ) from 0 to 1 according to a uniform

distribution. For each proportion to be simulated, n λ's were generated. A string of n 0's and 1's was then produced from the λ's: for $\lambda \leq \pi$, a 1 was generated, and for $\lambda > \pi$, a 0 was generated. The simulated outcome $\hat{\mu}$ was given by the sum of 1's in the string of n 0's and 1's. The simulated proportion ($\hat{\pi}$) was calculated by dividing $\hat{\mu}$ by n. Each proportion $\hat{\pi}$ within each data set was estimated by using the above procedure. For each experimental design, two different situations were investigated for estimating values of power: the difference between treatments was fixed and the sample size was allowed to vary; the sample size was fixed and the difference between treatments was allowed to vary. For type I error, all π's were fixed to 0.5 and sample size was allowed to vary. For each sample size or treatment difference of a given experimental design, 1000 data sets were produced and analyzed individually using different statistical analysis techniques and the results were reported as the percentage of times H_o was rejected at the 0.05 level of significance. The significance in logistic regression analysis was determined by the drop in deviance (Baker and Nelder 1978) while it was determined by the F-ratio when arcsine transformed or untransformed data were analyzed. Details for each experimental design are given below.

2x2 contingency tables

Each individual data set had two treatment groups with only one replicate for a total of two $\hat{\pi}$ values. The first series of data sets was simulated by fixing π_1 = 0.5 and π_2 = 0.2 and varying the sample size n from two to 100. A second series of data sets was made by fixing π_1 = 0.1, n = 10 and allowing π_2 to vary from 0.1 to 0.95. In a the third series of data sets, π_1 = 0.1, n = 50, and π_2 varied

from 0.1 to 0.95. In the last series, $\pi_1 = \pi_2 = 0.5$ and n varied from five to 200. Logistic regression analysis, equivalent to *G* test in this case, was compared with the Chi-square test for independence (Zar 1984).

ANOVA, two treatment groups, two replicates

Each individual data set had two treatment groups with two replicates each for a total of four $\hat{\pi}$ values. The first series of data sets was simulated by fixing $\pi_1 = 0.2$ and $\pi_2 = 0.5$ and varying the sample size from two to 100. A second series of data sets was made by fixing $\pi_1 = 0.1$, n = 10 and allowing π_2 to vary from 0.1 to 0.90. In a the third series of data sets, $\pi_1 = \pi_2 = 0.5$ and n varied from three to 50. Data were analyzed to test for $\pi_1 = \pi_2$ using logistic regression and using classical ANOVA with arcsine transformed data as well as untransformed data (Baker and Nelder 1978).

ANOVA, five treatment groups, five replicates

Each individual data set had five treatment groups with five replicates each for a total of 25 $\hat{\pi}$ values. The first series of data sets was simulated by fixing $\pi_2 = \pi_3 = \pi_4 = \pi_5 = 0.5$ and $\pi_1 = 0.2$ and varying the sample size from two to 14. A second series of data sets was made by fixing $\pi_2 = \pi_3 = \pi_4 = \pi_5 = 0.5$, n =10, and allowing π_1 to vary from 0.5 to 0.1. In a the third series of data sets, $\pi_1 = \pi_2 = \pi_3 = \pi_4 = \pi_5 = 0.5$ and n varied from two to 14. Data were analyzed to test for $\pi_1 = \pi_2 = \pi_3 = \pi_4 = \pi_5$ using logistic regression and using classical ANOVA with arcsine transformed data as well as untransformed data (Baker and Nelder 1978).

Simple linear regression

Each individual data set had three x values (0, 1, and 2) with one π_i value for each x_i value for a total of three $\hat{\pi}_i$ values. The first series of data sets was simulated using π_0=0.3, π_1=0.5, and π_2=0.7 and varying the sample size from five to 50. A second series of data sets was made by fixing $\pi_1 = 0.5$, n = 10, and allowing the difference $\pi_1 - \pi_0$ and $\pi_2 - \pi_1$ to vary simultaneously from 0.0 to 0.45. In a the third series of data sets, $\pi_0 = \pi_1 = \pi_2 = 0.5$ and n varied from two to 50. Data were analyzed to test for slope = 0 using logistic regression and using classical regression for arcsine transformed data as well as untransformed data (Baker and Nelder 1978).

Multiple regression

Each individual data set had two treatment groups with three x values (0, 1, and 2) each. There was one π_i value for each x_i value of any group for a total of six $\hat{\pi}_i$ values. The first series of data sets was simulated using $\pi_0 = \pi_1 = \pi_2 =$ 0.5 in group one compared to π_0=0.5, π_1=0.7, and π_2=0.9 in group two. The sample size varied from five to 70. A second series of data sets was made by fixing $\pi_0 = \pi_1 = \pi_2 = 0.1$ in group one and in group two $\pi_0 = 0.1$, n = 10, and the difference $\pi_2 - \pi_0$ was allowed to vary from 0.0 to 0.88 while the difference $\pi_1 - \pi_0$ was set at half the difference $\pi_2 - \pi_0$. In a the third series of data sets, π_0 $= \pi_1 = \pi_2 = 0.5$ for both groups and n varied from two to 50. Data were analyzed to test for equality of slopes between group one and two given a common intercept. Logistic regression and classical regression for arcsine transformed data as well as untransformed data were used (Baker and Nelder 1978).

RESULTS

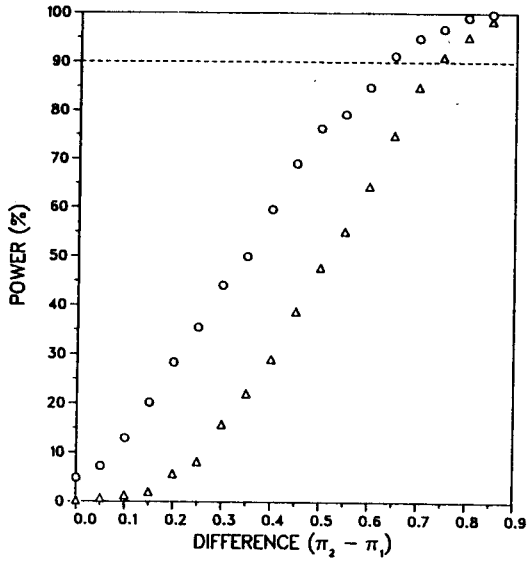
2x2 contingency tables

Logistic regression, equivalent in this case to a *G* test for contingency table, was a more powerful technique than Chi-square test (Fig 17). However, with proportions based on $n < 50$, the type I error using logistic regression varied from 4 to 9 % while it stayed below 5% with Chi-square analysis.

ANOVA, two treatments, two replicates

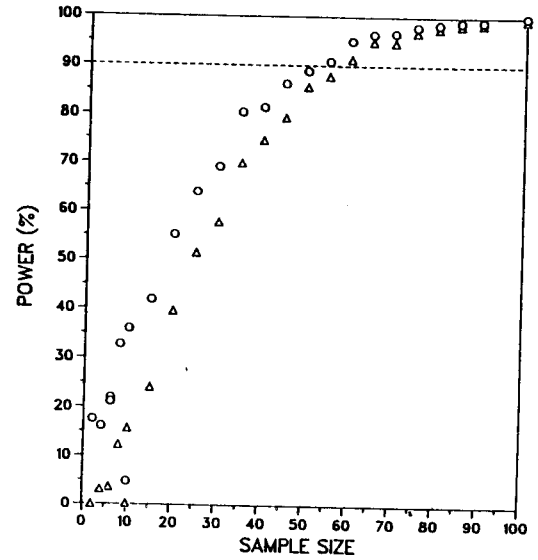
It took four times more samples to reach 90% power with arcsine transformed using classical ANOVA than with logistic regression (Fig. 18). With $n = 10$, logistic regression could detect a difference of at least half the size of the difference detected by arcsine transformed data using classical ANOVA. Type I error was above 6% with arcsine transformed data only when $n = 3$ and $n = 5$. It was not above 6% for logistic regression.

Figure 17. The effect of sample size and difference between two groups on power and type I error when two proportions (π_{1-2}) with one replicate each were simulated and tested for equality using logistic regression and Chi-square analyses. The sample size represents the number of sampling units used for estimating π_i. For each value of the abscissa, 1000 data sets were generated and analyzed separately using the two analysis techniques. The percentage of times significance was found at the 0.05 level was reported as type I error or power depending on the validity of H_0.



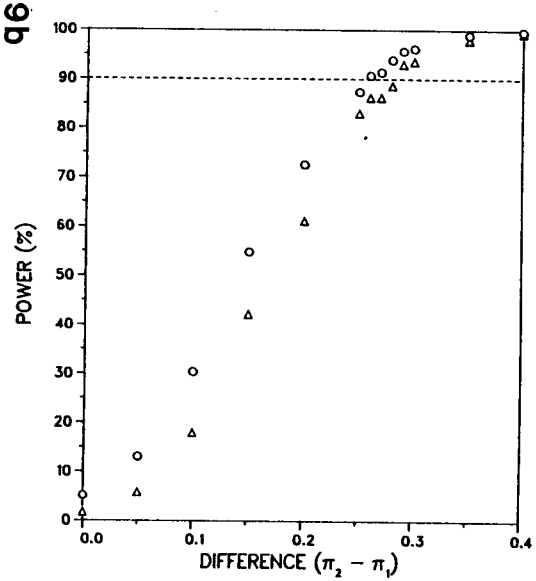
2 x 2 CONTINGENCY TABLE:
π_1 = 0.10,
π_2 = 0.10 TO 0.95,
AND SAMPLE SIZE = 10.

LEGEND
o = LOGISTIC REGRESSION
Δ = CHI-SQUARE TEST



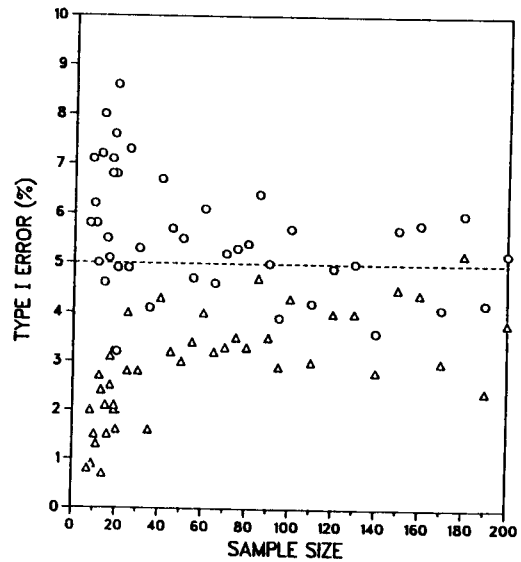
2 x 2 CONTINGENCY TABLE:
π_1 = 0.50 AND
π_2 = 0.20.

LEGEND
o = LOGISTIC REGRESSION
Δ = CHI-SQUARE TEST



2 x 2 CONTINGENCY TABLE:
π_1 = 0.10,
π_2 = 0.10 TO 0.95,
AND SAMPLE SIZE = 50.

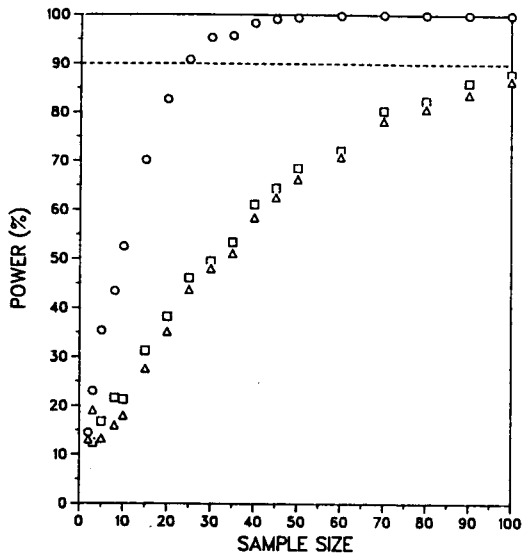
LEGEND
o = LOGISTIC REGRESSION
Δ = CHI-SQUARE TEST



2 x 2 CONTINGENCY TABLE:
π_{1-2} = 0.50.

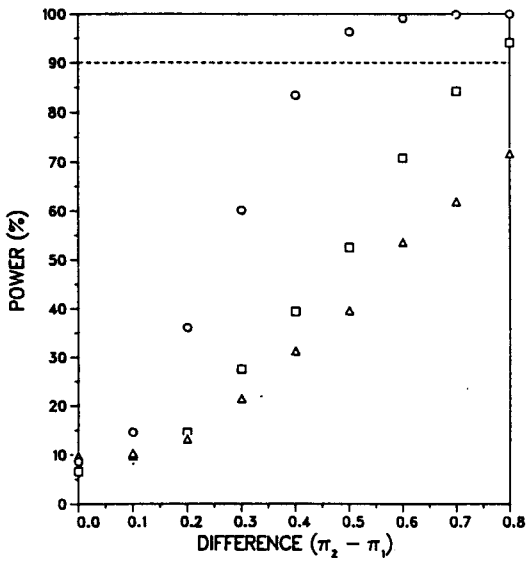
LEGEND
o = LOGISTIC REGRESSION
Δ = CHI-SQUARE TEST

Figure 18. The effect of sample size and difference between two groups on power and type I error when two proportions ($\pi_{1\text{-}2}$) with two replicates each were simulated and tested for equality using logistic regression and ANOVA with arcsine transformed or untransformed data. The sample size represents the number of sampling units used for estimating π_i of each replicate. For each value of the abscissa, 1000 data sets were generated and analyzed separately using the different analysis techniques. The percentage of times significance was found at the 0.05 level was reported as type I error or power depending on the validity of H_o.



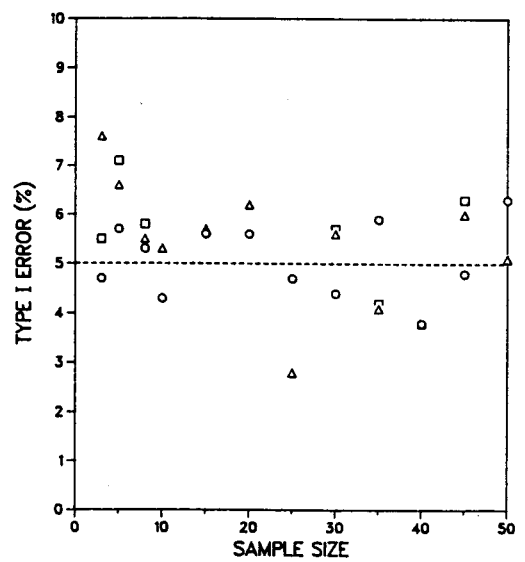
COMPLETELY RANDOMIZED DESIGN:
2 GROUPS,
2 REPLICATIONS,
π_1=0.20, AND
π_2=0.50.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



COMPLETELY RANDOMIZED DESIGN:
2 GROUPS,
2 REPLICATIONS,
π_1=0.10,
π_2=0.10--0.90,
AND SAMPLE SIZE = 10.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



COMPLETELY RANDOMIZED DESIGN:
2 GROUPS,
2 REPLICATIONS,
π_{1-2}=0.50.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA

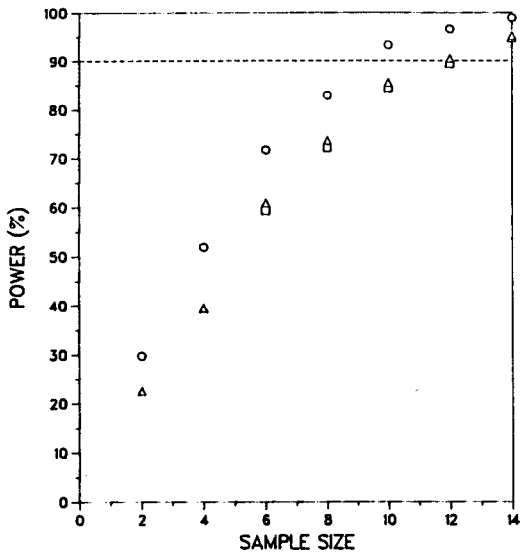
ANOVA, five treatments, five replicates

Using logistic regression, a power of 90% was reached with n = 9, while it took n = 12 to reach this power level with arcsine transformed data and classical ANOVA (Fig. 19). With n = 10, a proportion difference of 0.25 in one of the five treatments could be detected with a 90% power while the difference had to be at least 0.30 to be detected with the same power when classical ANOVA with arcsine transformed data were used. With n = 2, type I error was 8% when logistic regression was used while it was 5% when arcsine transformed data and classical ANOVA were used. With $n \geq 4$, both methods of analysis had a type I error of 5±1%.

Simple linear regression

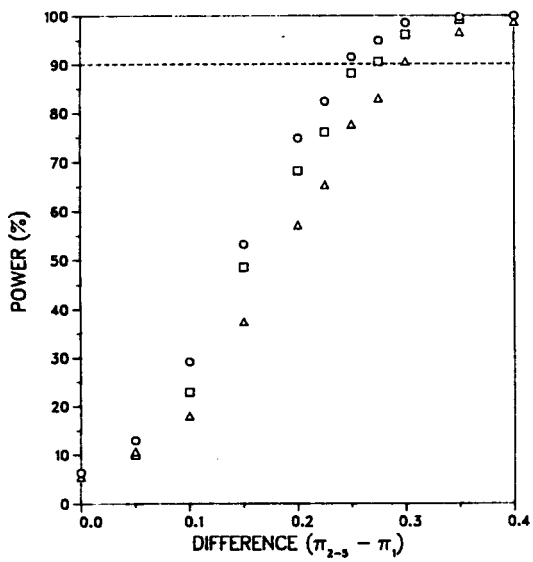
With n =30, 90% power was reached using logistic regression while the power was 15% using classical regression with arcsine transformed data (Fig 20). A slope of 0.35 could be detected with 90% power using logistic regression while the same slope was detected with a 15% power using classical regression with arcsine transformed data. The type I error using logistic regression was above 10% with $n < 5$ and, with arcsine transformed data and $n < 10$, the type I error was above 6%.

Figure 19. The effect of sample size and difference between one group (π_1) and the other four groups ($\pi_{2\text{-}5}$) on power and type I error when five proportions ($\pi_{1\text{-}5}$) with five replicates each were simulated and tested for equality using logistic regression and ANOVA with arcsine transformed or untransformed data. The sample size represents the number of sampling units used for estimating π_i of each replicate. For each value of the abscissa, 1000 data sets were generated and analyzed separately using the different analysis techniques. The percentage of times significance was found at the 0.05 level was reported as type I error or power depending on the validity of H_o.



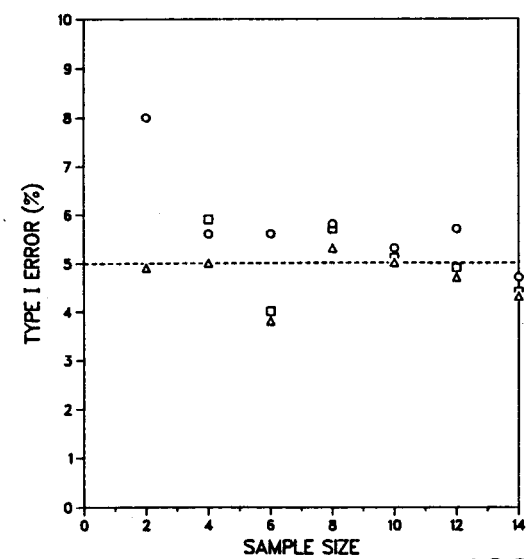
COMPLETELY RANDOMIZED DESIGN:
5 GROUPS,
5 REPLICATIONS,
π_1=0.20, AND
π_{2-5}=0.50.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



COMPLETELY RANDOMIZED DESIGN:
5 GROUPS,
5 REPLICATIONS,
π_1=0.10 TO 0.50,
π_{2-5}=0.50,
AND SAMPLE SIZE = 10.

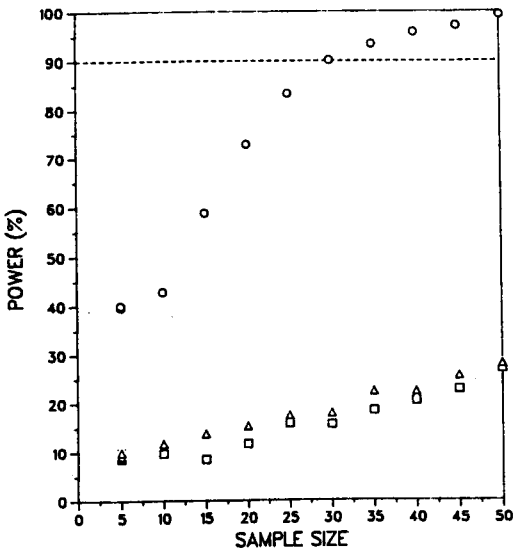
LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



COMPLETELY RANDOMIZED DESIGN:
5 GROUPS,
5 REPLICATIONS,
π_{1-5}=0.50.

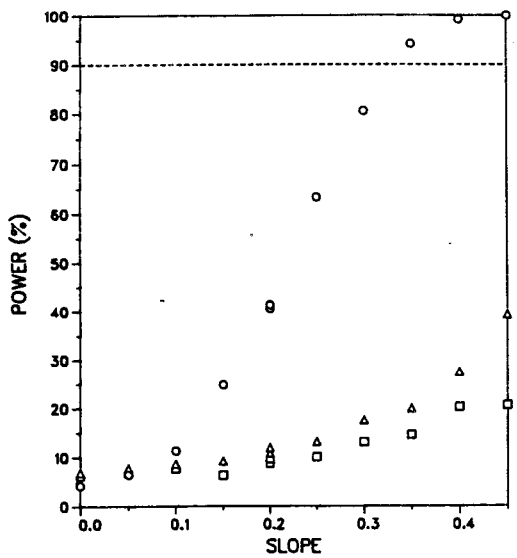
LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA

Figure 20. The effect of sample size and increment in slope on power and type I error when one replicate proportion π_i for each x_i value were simulated and tested for slope = 0 using logistic regression and classical regression with arcsine transformed or untransformed data. The sample size represents the number of sampling units used for estimating π_i at a given x value. For each value of the abscissa, 1000 data sets were generated and analyzed separately using the different analysis techniques. The percentage of times significance was found at the 0.05 level was reported as type I error or power depending on the validity of H_o.



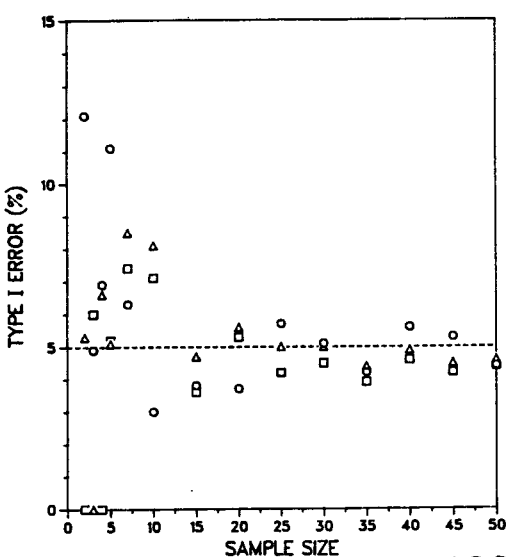
SIMPLE LINEAR REGRESSION:
3 EQUIDISTANT X–VALUES,
π_0=0.30,
π_1=0.50, AND
π_2=0.70.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



SIMPLE LINEAR REGRESSION:
3 EQUIDISTANT X–VALUES,
π_0=0.50 TO 0.05,
π_1=0.50,
π_2=0.50 TO 0.95,
AND SAMPLE SIZE = 10.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



SIMPLE LINEAR REGRESSION:
3 EQUIDISTANT X–VALUES,
π_0=0.50,
π_1=0.50, AND
π_2=0.50.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA

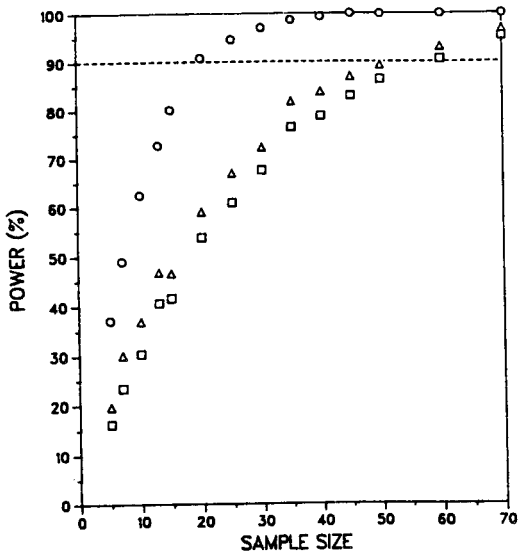
Multiple regression

A 90% power was reached with n = 20 with logistic regression while it took a sample of 50 to reach the same power level with classical regression (Fig. 21). A difference in slope of 0.27 could be detected with 90% power using logistic regression while the difference had to be above 0.4 to be detected with the same power when classical regression was used. With $n < 5$, the type I error was above 8% using logistic regression while it was always at 5±1% using classical regression with arcsine transformed data.

DISCUSSION

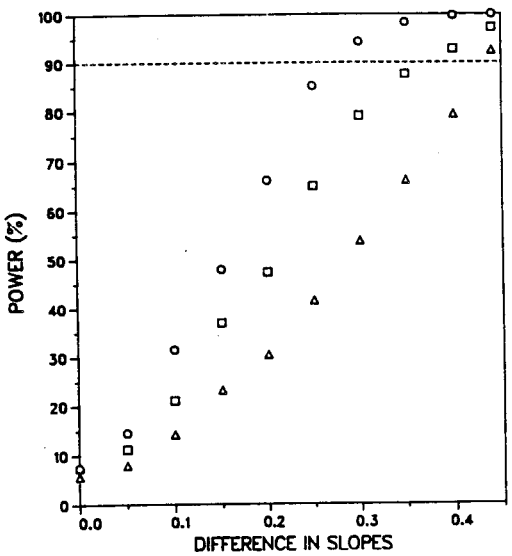
The power of logistic regression was consistently higher than in any other statistical analysis technique tested. One of the smallest differences in power was observed with five treatment groups and five replicates. In this case a power of 90%, the recommended level of power for an experiment, was reached with $n = 9$ with logistic regression compared to $n = 12$ with arcsine transformed data. If one considers n as being the number of plants needed to estimate a proportion in a given replicate, 225 plants would be needed in an experiment if logistic regression was used while 300 plants would be necessary to achieved the desired level of power with arcsine transformed data, a 25% reduction in sample size. With five treatments and replicates and with n = 10, the difference in one treatment detectable with a 90% probability was reduced by 17% when logistic regression was used compared to arcsine transformed data.

Figure 21. The effect of sample size and difference in slope between two curves on power and type I error when one replicate proportion π_i for each x_i value of curve one or curve two were simulated and tested for equality of slopes using logistic regression and classical regression with arcsine transformed or untransformed data. The sample size represents the number of sampling units used for estimating π_i at a given x_i value and for a given curve group. For each value of the abscissa, 1000 data sets were generated and analyzed separately using the different analysis techniques. The percentage of times significance was found at the 0.05 level was reported as type I error or power depending on the validity of H_o.



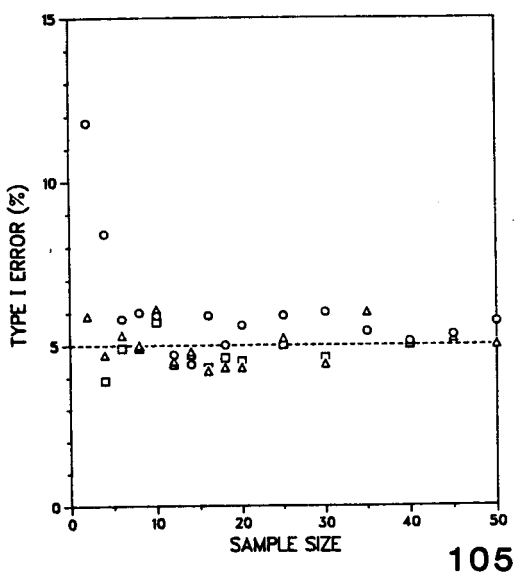
MULTIPLE REGRESSION:
3 EQUIDISTANT X–VALUES,
2 CURVES WITH INTERCEPTS AT 0.50
$SLOPE_1$=0.00, AND
$SLOPE_2$=0.20.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



MULTIPLE REGRESSION:
3 EQUIDISTANT X–VALUES,
2 CURVES WITH INTERCEPTS AT 0.10,
$SLOPE_1$=0.00, AND
$SLOPE_2$=0.00–0.44.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA



MULTIPLE REGRESSION:
3 EQUIDISTANT X–VALUES,
2 CURVES WITH
INTERCEPTS = 0.50 AND
SLOPES = 0.00.

LEGEND
○ = LOGISTIC REGRESSION
△ = ARCSINE TRANSFORMATION
□ = UNTRANSFORMED DATA

In contingency tables, the type I error using logistic regression was higher than it should be with sample sizes below 50 while the Chi-square test tended to be more conservative and gave a type I error lower than the significance level. It appears that Chi-square would be preferable to logistic regression for proportions estimated with $n < 50$. In all other designs tested, logistic regression had a type I error higher than expected only when sample size was smaller than five. Therefore, as reported by Schaefer (1983), the maximum likelihood estimates used in logistic regression can be biased when sample size is small.

It appears that for the ANOVA and regression designs tested, logistic regression would be preferable to arcsine transformation with classical analysis techniques if the proportions are being estimated with $n \geq 5$. Except for the ANOVA with five replicates and treatments, the designs tested here were extremes with respect to low replicate number thus it is unlikely that one would find too many situations where the type I error would be committed with $n \geq 5$. By using a more powerful technique, one reduces the probability of committing type II error, *i.e.* 100% - power or the chance of accepting the null hypothesis when H_0 is false. The inference of absence of significance using an analysis technique with 90% power (type II error = 10%) is more meaningful than it is for one with 50% power (type II error = 50%), *i.e.* one's chance of not detecting a real difference with 90% power is reduced by five fold when compared to 50% power.

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