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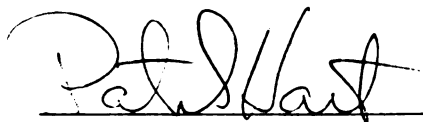
The Influence of Plant Age on the Infection of
Soybean Plants by Phytophthora megasperma f. sp.
glycinea and Survival on Alternate Hosts.

presented by

Mariflor Stella Avila Silva

has been accepted towards fulfillment
of the requirements for

Master's degree in Plant Pathology



Major professor

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**THE INFLUENCE OF PLANT AGE ON THE INFECTION OF SOYBEAN PLANTS
BY PHYTOPHTHORA MEGASPERMA F. SP. GLYCINEA AND SURVIVAL ON
ALTERNATE HOSTS**

By

Mariflor Stella Avila Silva

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Submitted to
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ABSTRACT

THE INFLUENCE OF PLANT AGE ON THE INFECTION OF SOYBEAN PLANTS BY PHYTOPHTHORA MEGASPERMA F. SP. GLYCINEA AND SURVIVAL ON ALTERNATE HOSTS

By

Mariflor Stella Avila Silva

Plants 2 to 50 days old were inoculated with P. m. glycinea. Inoculated 2, 4 and 6 days old plants were killed within 8 days of inoculation but plants up to 36 days old showed increased levels of resistance. Susceptibility increased for plants 43 and 50 days old at the time of inoculation. Increased susceptibility was correlated with the appearance of the first flowers. Increased susceptibility in older plants does not agree with field observations and may suggest other mechanisms of disease escape.

Plants of susceptible and resistant soybean cultivars, wheat, alfalfa, dry bean and corn were inoculated with P. m. glycinea to study the possible survival of the pathogen in non-hosts. Varying numbers of oospores were identified in the roots of both susceptible and resistant cultivars of soybeans as well as in non-host plants. After soybeans, dry bean roots supported the highest number of oospores, without showing apparent symptoms. The design of future rotations may have to take these results into account.

To: Ing Agr Nicolas Chebataroff
who introduced me into the agricultural
research world.

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INTRODUCTION

INTRODUCTION

Phytophthora root rot or root and stem rot of soybeans caused by the fungus Phytophthora megasperma (Drechs.) f. sp. glycinea Kuan and Erwin (= Phytophthora megasperma Drechs. var. sojae Hildeb.), is one of the most destructive soilborne diseases of soybean (*Gycine max* (L.) Merr.) and the only severe Phytophthora disease of a major grain crop in the United States. The disease was first observed in Indiana in 1948 (Schmitthenner, 1985) and now occurs in most soybean producing areas of the U.S. and Canada. Based on a phone survey of soybean pathologists, Schmitthenner (1985), reported an estimate of approximately 16 million acres was infested with P. megasperma f. sp. glycinea and subject to damage under conditions conducive to root rot in the United States and Canadá.

References based on field observations said that the pathogen may attack susceptible soybean plants in all stages of growth. The disease development is favored by poorly drained soils and wet weather. Symptoms of the disease are pre-and postemergent damping off of seedlings, root and stem rot, and stunting, wilting or death of older plants.

Several studies (Herr, 1957, Paxton et al, 1969, Meyer et

al, 1972 and Jimenez et al, 1980), reported that susceptibility of soybean plants to P. megasperma f. sp. glycinea decreased as the plants became older and that mean shoot lengths, foliar and root weight were less than the control in inoculated plants. These results were found in plants up to three weeks old. Studies on the influence of plant age on infection by this pathogen in older plants have not been reported. During middle of the 1991 growing season (June and July) moisture conditions were favorable for severe disease development in the Michigan soybean crop, but almost no disease was reported that year. The question was: do plants develop resistance as they age or are other factors responsible for the lack of mid-season infection? The role of plant age on susceptibility to P. megasperma f. sp. glycinea is investigated in chapter 1.

Isolates of P. megasperma from soybeans that are pathogens of hosts other than soybeans have been described since 1961 and based on these studies, rotation of soybeans with other crops (wheat, lucerne, corn), was not considered a problem. However, the possible survival of P. megasperma f. sp. glycinea in alternate or non-hosts in which it is not pathogenic but where zoospores could infect and produce oospores in the roots, has not been studied. The possibility of these alternate hosts being a source of infection for the next susceptible soybean crop in the same field, is studied in Chapter 2.

LITERATURE REVIEW

LITERATURE REVIEW

History of the disease.

Phytophthora root rot of soybeans was first noted as a new disease of unknown etiology in Indiana in 1948 and similar root rot symptoms were found in Ohio in 1951. Symptoms were originally thought to be caused by Fusarium or Diaporthe. Phytophthora was first associated with soybeans in North Carolina and Ohio in 1954 (Schmitthenner, 1985). The first reports of this disease were from Ohio and North Carolina in 1955 (Skotland, 1955; Suhovecky, 1955; Suhovecky et al, 1955) and identified as a disease caused by Phytophthora cactorum (Leb & Cohn) Schroet. Later, Kaufmann et al (1958), found an undescribed species of Phytophthora associated with root and stem rot of soybeans in Illinois; they published the first comprehensive report of the disease and proposed the name Phytophthora sojae, due to differences in morphology, pathogenicity and growth rate with the closely related species P. cactorum and P. megasperma. In 1959, Hildebrand changed the name to Phytophthora megasperma Drechs. var. sojae Hideb. This name was valid until 1980, when the fungus was reclassified as P. megasperma f. sp. glycinea by Kuan et al., after extensive studies of the host range and oospore size.

They concluded that oogonial size of *P. megasperma* isolates from different hosts overlap and was, therefore, unsuitable for a variety separation. However, soybean and alfalfa isolates of *P. megasperma* had a sufficiently distinct host range to place them into two forma speciales (*P. megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis*), separated from *P. megasperma* found in other hosts.

Symptoms.

Seeds and germinated seeds often rot before emergence, thereby reducing stands. Young plants after emergence are very susceptible and they often wilt and die. Older plants may be stunted and gradually die throughout the growing season (Hart et al., 1981). Stems of plants attacked in the primary-leaf stage first appear water-soaked, then the leaves wilt and the entire plant dies and turns brown. The first symptom in older plants is a yellowing and wilting of the lower leaves, followed by yellowing and/or wilting of the entire plant. Leaves commonly remain attached for a week or more on dead plants, (Hart et al., 1981). Most lateral roots die and the tap root turns dark brown. Chlorotic areas appear between the veins and along the margins of the leaves and a brown canker advances up the stem, frequently as far as the node of the second or third trifoliate leaf. At times lesions may be confined entirely to the stem, with lower portions of the stem and the roots having no perceptible symptoms (Kaufmann et al., 1958). Internally the discoloration extends through the

cortex to the vascular tissues which may also be involved (Hildebrand, 1959). Dr. Scott (Purdue University), distinguished two phases of Phytophthora root rot: the seedling phase from the time soybeans are planted to approximately the first trifoliate stage of development and the root rot phase, which can occur any time after emergence. Symptoms of the seedling phase may be easily confused with Pythium seedling blight, Rhizoctonia root rot, water damage, poor germination, herbicide damage or other causes. As a result, Phytophthora root rot often goes undiagnosed until stands are severely reduced, when it is too late to positively identify the pathogen. When infected seedlings are examined early, the hypocotyl and or roots appear shrunken and darkly discolored by a relatively dry rot. The root and stem rot phase lead to a slow leaf yellowing, wilting and death, or stunted, less vigorous plants.

Favorable conditions.

Environmental factors play an important role on infection and disease severity of Phytophthora root and stem rot of soybeans. The most important of these factors are soil compaction, soil moisture and soil temperature. Conditions favorable for infection occur most often in heavy, compacted clay soils with poor drainage. Disease incidence and the number of dead plants increased with compaction, while seed weight and total number of plants emerged decreased (Moots et al, 1988). Periods of high soil moisture, rainfall or ponded

water favor the disease. Even after roots have been invaded by *P. megasperma* f. sp. *glycinea* (hereafter referred to as *P. m. glycinea*) unless wet conditions are maintained, root regrowth occurs (Moots et al, 1988). Once soils dry, the soybean seedlings are capable of regenerating a healthy root system. The disease is most severe in years with heavy rainfall early in the growing season and is most destructive in low, poorly drained portions of the field (Kittle et al., 1979). In greenhouse experiments, Klein (1959), found that the percentage of diseased plants increased with the length of the wet period of the soil before planting. Soil temperature may be the most important environmental factor influencing disease severity (Kittle et al., 1979). The optimum soil temperature for infection are 27°C to 33°C for seedlings and young plants and 25 to 30°C for older plants, but infection can occur at soil temperatures as low as 15°C (Kittle et al, 1979). Greatest root loss in *P. m. glycinea* infested soils occurred at lower temperatures than at the optimum. At low temperatures *P. m. glycinea* may have greater ability to attack and destroy roots than the soybean ability, to replace them. As soil temperature increases above 15°C, the soybean plants became more competitive or *P. m. glycinea* becomes less aggressive or both (Kittle et al., 1979).

The pathogen.

P. megasperma f.sp. *glycinea* is homothallic; mycelium and sporangia are diploid. Meiosis occurs in antheridia and

oogonia and nuclear fusion takes place in the oogonium, which forms a diploid oospore. Germination of oospores results in mycelium and sporangia. Zoospores are released from maturing sporangia and are motile in free water; zoospores are attracted to and aggregate around germinating seeds, young roots or exudates of older roots (Schmitthenner, 1985).

Mycelial growth of the fungus is typical of that in the Genus Phytophthora; hyphae exhibit characteristic right-angle branching with slight constrictions at the base of the branches. Oogonia are large, with a mean diameter of 41 μm , and antheridium are mostly paragynous or occasionally amphigynous. (Schmitthenner, 1985). They are formed very quickly (three to four days) in culture plates on lima bean agar when incubated at 24°C. Oospores also form readily in culture and in diseased tissue (Schmitthenner, 1985). The spherical oospores, have thick, smooth walls and nearly fill the oogonia. A total of 194 oogonia and oospores were measured with an average of 36.9 μ and 31.4 μ respectively (Kaufman et al., 1958). They also measured the thickness of the oospore walls and found an average of 2.4 μ . Sporangia were formed in culture medium only when wefts of mycelium were placed in Petrie's solution for 6 to 8 hours at 25°C (Skotland, 1955). Sporangia also form readily if mycelia are washed repeatedly in water or Chen-Zentmyer salt solution (Schmitthenner, 1985). Sporangia have obpyriform shape (mean length 40 μm , mean width 28 μm), are non-papillate and

proliferous (new sporangium form inside one that has germinated) (Schmitthenner, 1985). Five to 20 biflagellate zoospores are formed within each sporangium and released through the apical end. Encysted zoospores were approximately 13μ in diameter and germinated directly (Skotland, 1955, Kaufmann et al., 1958).

Ecology and epidemiology.

P. megasperma f. sp. glycinea survives as oospores in crop residues and in soil for many years but does not grow competitively or colonize soil debris (Schmitthenner, 1985). The fungus can not be demonstrated in soil immediately after freezing or storage for long periods at 3°C, indicating that mycelium, sporangia and zoospores do not survive cold temperatures. If overwintered soil is allowed to incubate for one week at 25°C under suitable moisture conditions, the fungus can be readily demonstrated with the leaf disk bait technique (Schmitthenner, 1985). This data indicates that P. megasperma f. sp. glycinea survives as a resistant oospore that germinates when dormancy is broken and when temperature and moisture are suitable. It is not known exactly what factors break the dormancy of oospores and what minimum soil saturation times at different temperatures favor the germination of oospores and formation of sporangia. The relative importance of primary and secondary inoculum for infection has not been established, and it is known that extended rotations with a nonhost do not eliminate the

pathogen (Schmitthenner, 1985).

CHAPTER 1.

CHAPTER 1

Effect of plant age on the infection of soybean plants by Phytophthora megasperma f. sp. glycinea.

INTRODUCTION AND LITERATURE REVIEW

The susceptibility of many plants to various diseases changes as the plant or specific plant tissue becomes older. Herr (1957) reported that 1-3 weeks old soybean plants were more susceptible to Phytophthora cactorum (Leb & Cohn) Schroet (= Phytophthora f. sp. glycinea) than older plants. He also reported that the rate at which the inoculated plants died decreased progressively with increasing plant age. Paxton et al (1969), found that seven day-old plants were uniformly susceptible, but they gradually changed to resistant as up to 21 days old. Five days following inoculation, all highly susceptible plants were dead. Several plants, especially those 14 days old at inoculation, showed brown stem cankers at this time. Some of these cankers eventually enlarged to kill the plants, while others became limited and static, especially in the plants inoculated when they were 21 days old. Most of the plants 14 days old at inoculation eventually succumbed to

infection, but generally at a much slower rate than the plants inoculated when they were 7 days old. They found that there are at least two types of disease resistance in soybeans: resistance in young plant tissue (0-2 weeks old) in which the production of phytoalexins plays an important role; and resistance in older plant tissue (more than 2 weeks old) recognized by woody stem tissue and greatly reduced phytoalexin production (Paxton et al, 1969). Mean lengths and weight of plants inoculated up to 8 days of age were significantly less ($p=0.05$) than the control. Thereafter, differences narrowed and were not significant, even though root infection of inoculated seedlings was still evident. Plants inoculated at 16 days of age or older showed increased resistance and the maximum effects of disease were expressed when younger plants were inoculated (Jimenez et al. 1980). Phytophthora megasperma var. sojae reduced the root system of soybeans in the presence or absence of stem lesions. Susceptible cultivars grown in infested soils in the greenhouse and field had no stem lesions, but plant height and yields were significantly less than similarly treated resistant cultivars. The pathogen can reduce the root system and dry weight of above ground parts of soybean seedlings with or without the production of stem lesions (Meyer et al, 1972). Ward et al (1981) reported that in etiolated 6-day-old soybean hypocotyls, resistance to Phytophthora megasperma var. sojae increased from the top to the bottom. The current accepted

name for this pathogen is Phytophthora megasperma f. sp. glycinea.

The research presented in this chapter examines the relationship of plant age to overall plant growth and looks at the effect of P. megasperma f. sp. glycinea. on plants older than previously reported.

MATERIALS AND METHODS

Growth chamber experiment.

Preparation of plants.

Soybean seeds of the cultivar Conrad were surface sterilized in 0.5 % NaOCl for 20 minutes, washed three times with sterilized distilled water, and germinated in rolled up moistened paper towels for two days at room temperature. (Seeds were germinated this way to reduce variability; germination rates were low for the seed lot used). Seedlings were transplanted at different times so that plants of different ages were all inoculated at the same time. The seedlings were transplanted when they were about 1.5 cm in length, into aluminum trays 49 cm long, 30 cm wide and 8 cm high, using Bacto Professional Planting Mix (Michigan Peat Co.). Five seedlings of a given age were planted in each tray, in rows 5 cm apart. Plants were fertilized with Ra-Pid-Grow plant food (23-19-17) from Chevron Chemical Company,

using a concentration of 4.14 g per liter of water, applying 420 cc of this mix per tray. Plants were fertilized the first day of planting and 10 days later. The temperature was maintained at 20°C with a 16 hr photoperiod. Each planting was replicated five times. Treatments consisted of plants inoculated at 2, 4, 6, 8, 10, 12, 14 and 21 days of age.

Zoospore production

Comparison of zoospore production for two races of Phytophthora megasperma f. sp. glycinea.

Races 1, 3, 4 and 7 were kindly supplied by Dr. Schmitthenner (Ohio State University). According to Lockwood et al (1978), races 1, 3, 4 and 6 predominate in Michigan soybean fields. To select one race to work with, a comparison of zoospore production was done. Races 3 and 7 were not used because of consistently poor zoospore production. The method of Eye et al (1978) was used. Seven to 12 day-old cultures of Phytophthora megasperma f. sp. glycinea races 1 and 4, grown on lima bean agar in the dark at 24°C, were washed, 4x(20 min/wash) with a 10% salt solution (Dr.Lockwood, personal communication; Aphanomyces Replacement Solution: 2.94g CaCl₂ 2H₂O, 2.47 g MgSO₄. 7H₂O, 0.75 g KCl and 1000 ml of distilled water), flooded with 10 ml of the same solution and incubated overnight in the dark at 24°C. After 1-20 hr of incubation, zoospores were collected and counted. Each race was grown on three replicated plates and six counts of zoospores were made

with a hemacytometer for each plate. The race of P. megasperma f. sp. glycinea producing the most zoospores in the shortest time was selected for all future experiments.

Inoculation.

All inoculations were made with P. m. glycinea race 4, (2.7×10^4 zoospores/ml) when the plants were 2, 4, 6, 8, 10, 12, 14 and 21 days old. Each plant was inoculated with a syringe by injecting 2.5 ml of the suspension around the base of the plant into the soil. After inoculation, the trays were flooded with water, and more water was added thereafter as needed to prevent the soil from drying out. Controls consisted of a single tray of 2 to 21 day-old non-inoculated plants.

Disease assessment.

One week after inoculation, the plants were evaluated for disease symptoms using the following scale: 1 = healthy plants; 2 = small brown lesions on the base of the stems (< 1 cm long); 3 = enlarged brown lesions (> 1 cm long) on the stem; 4 = enlarged brown lesions on the stems and wilting of the lower leaves; 5 = severe wilt of all the plants; 6 = plant died. In addition, the number of plants remaining alive 20 days after inoculation was determined, and these plants were harvested ten days later. Dry weights were determined for aerial parts of the plants by drying in an oven for one day at

45°C, and two days at 65°C. The roots were dried between paper towels at room temperature and then weighed.

Green house experiment.

The growth chamber experiment was repeated in the greenhouse, and expanded by using plants older than three-weeks-old.

Preparation of plants.

Soybean seeds of the cultivars Sloan (Susceptible) and Dassel (Resistant) were surface sterilized in 0.525% NaOCl for 20 minutes, washed three times with sterile distilled water, and germinated for two days in rolled up moistened paper towels at 24°C, as described previously. When the seedlings were about 1.5 cm in length, they were planted into Bacto Professional Planting Mix (Michigan Peat Co.) in 59 cm long 33 cm wide and 27 cm high plastic trays. Five seedlings per age were planted in each tray. Plants were fertilized twice, once one month after the first planting and once 12 days later, with Ra-Pid-Grow plant food (23-19-17) at a concentration of 4.0 g per liter of water; 0.84 L of that concentration was added each time, to each tray. There were four plantings (replications) made for each cultivar.

Zoospore production.

Zoospores of race 4 were produced as described in the growth

chamber experiment. Zoospores were counted by the microsyringe method of Ko, et al (1973). The method consists of counting the zoospores in one μl of a zoospore suspension. The zoospore concentration was determined by averaging the results of counting one hundred $1\text{-}\mu\text{l}$ samples.

Inoculation.

The inoculations were made when the plants were 5, 8, 15, 22, 29, 36, 43 and 50 days old, with a suspension of 7.1×10^3 zoospores/ml. The different age plants were all inoculated at the same time. The inoculations were made by pouring the zoospore suspension into eighteen holes made with 15 ml centrifuge tubes and equally spaced throughout the tray, always between rows of plants. To inoculate, the tubes were removed and 15 ml of the spore suspension was added into each hole. A total of 270 ml of zoospore suspension was added on each tray. Each treatment was replicated four times. Controls consisted of one tray of non-inoculated plants. After inoculation, the trays were maintained in a flooded condition for one week and then watered as needed.

Disease assessment.

Disease ratings were made 8 and 40 days after inoculation, and the number of plants remaining alive after 40 days were determined. For disease rating the following rating scales were used: For ratings 8 days after inoculation: 1 =

healthy plants; 2 = wilting of the lower leaves; 3 = brown discoloration of the stem only; 4 = brown discoloration of the stem and general wilting; 5 = brown discoloration, wilting, and some chlorosis and dying of the leaves; 6 = plant dead. For disease rating 40 days after inoculation: 1 = healthy plants; 2 = brown discoloration < 3 cm in length on the stem; 3 = brown discoloration >3 and <8 cm on the stem; 4 = brown discoloration > 8 cm on the stem; 5 = brown discoloration > 8 cm on the stem and chlorotic or dying leaves; 6 = plant dead. Different ratings were used because after 40 days wilting was not present and the most consistently observable difference was the length of the stem lesions.

Dry weight of aerial parts of the surviving plants was determined 40 days after inoculation. The samples were dried in a forced air oven for three days at 65°C and then weighed.

Analysis of data

All results were analyzed by two-way analysis of variance. ANOVA tables for each experiment are presented in the appendix. Differences among treatments were determined by Duncan's Multiple Range Test. A factorial analysis was not made because the different age plants were inoculated at the same time. All statistical analysis were made using MSTAT C statistical software package developed in the Crop and Soil Science department at MSU.

RESULTS

Growth chamber experiment

Zoospore production

Maximum production of zoospores occurred after 12 hr of incubation for race 4 and after 14 hr for race 1. Race 4 produced the highest number of zoospores (Fig 1). Therefore, race 4 of *P. m. glycinea* and a 12 hr incubation period were selected for future experiments.

Disease assessment

Inoculation of 2, 4 and 6 day-old plants resulted in mean disease ratings of 5.8, 5.8, and 5.6 respectively and almost all the plants died. Inoculation of the 8 to 14-days-old plants resulted in mean disease ratings between 1.6 - 2.6 and approximately 1-cm long lesions on the stems; they showed increased levels of resistance. Twenty one day- old plants at the time of inoculation showed very few foliar symptoms (Fig 2). Most of the plants inoculated at 2, 4 and 6 days of age were killed within one week. More plants remained alive when 8 days old at the time of inoculation, but all plants remained alive when inoculated 12 days of age or older (Fig 3). Statistical analysis (Two-way analysis of variance) was performed for disease rating and the percentage of living plants 20 days after inoculation (Table 1). There were highly significant differences ($P < 0.01$) among treatments but

not among trays. Disease rating and percentage of surviving plants were significantly different between plants inoculated at 2, 4 and 6 days of age, and those inoculated at 8 days of age and later (Duncan's multiple range test, $P=0.05$). The fresh root weight of inoculated plants was always lower than the control plants (Fig 4 and 5). Although plants inoculated at 21 days of age were symptomless, the fresh root weight was about 16% lower than the control and exhibited severe root rot (Fig 5, Table 2). Foliar dry weight of inoculated plants was always lower than the non-inoculated control plants (Fig 6). The foliar dry weight of plants inoculated at 21 days of age was only 54.2 % of the non-inoculated controls. Statistical analysis (two-way analysis of variance) of fresh root weight and foliar dry weight showed significant differences ($P < 0.01$) among treatments but not among blocks or trays (Table 2). Means to determine weight as a percent of the control plants were calculated from plants living 20 days after inoculation; this varied from one to twenty five plants depending on the age of the plants at inoculation. Statistical comparison may not be valid because of means being derived from a different number of plants.

Greenhouse experiment

Disease assessment

There was a trend for decreasing disease ratings eight days after inoculation, as plant age at the time of

inoculation increased up to 36 days, but then disease ratings increased again for plants inoculated at 43 and 50 days of age (Fig 7). Figs 8 and 9 show the inoculated plants, 8 and 40 days after inoculation. Statistical analysis of the data (Table A1; A refers to Appendix), showed highly significant differences among treatments ($P < 0.01$) but not among blocks or trays. Although disease ratings 40 days after inoculation were higher than at 8 days after inoculation, the disease ratings were not directly comparable because differences in the method of rating. Visually, however, the plants forty days after inoculation appeared less healthy than at eight days after inoculation (Figs 8 and 9). Plants 36 days old at inoculation did not show symptoms 8 days after inoculation but were severely diseased after 40 days. Plants older than 36 days old at the time of inoculation had high disease rating at both, 8 and 40 days, with many dead plants after 40 days. The differences in disease ratings among plants of different ages was less 40 days after inoculation than at 8 days after inoculation. See Appendix (Table A1).

Disease ratings for the cultivar Dassel were 1 for all the treatments because this cultivar did not show symptoms at 8 days or 40 days after inoculation.

Foliar dry weight of the cultivars Sloan and Dassel.

Foliar dry weight of inoculated plants of the cultivar Sloan showed greater differences from the control as the

plants got older (Fig 11). Differences between inoculated and non-inoculated plants were more evident for plants older than 22 days at the time of inoculation, except 36 days. Statistical analysis of dry weight as a percent of the control for the susceptible cultivar Sloan, showed significant differences among treatments ($P < 0.01$) but not among blocks or trays (Table A 2). Although there were significant differences in the foliar dry weight of plants inoculated at different ages ($P < 0.01$), there were no readily apparent trends except that plants 43 and 50 days old at inoculation had consistently reduced foliar dry weight (Fig 11). There was however, a trend toward a higher number of surviving plants up to 36 days of age and then a decrease for plants 43 and 50 days old at inoculation (Fig 10). The resistant cultivar Dassel did not show significant differences or disease symptoms.

DISCUSSION

The results of the growth chamber experiment were similar to previous reports (Herr, 1957, Paxton et al., 1969). That is, as plants up to 21 days of age were inoculated, they appeared to be more resistant to disease development. The greenhouse results are new and suggest that plants older than 36 days at the time of inoculation have increased susceptibility. Also plant resistance to infection seemed to increase up to thirty- six day-old plants. The disease ratings 40 days after inoculation showed similar relative results as at 8 days after inoculation, but with an overall higher disease rating. These results suggest that older plants do not become completely resistant, but the rate of disease development may be slower in older plants. Plants inoculated at 43 and 50 days-of-age, showed very high levels of infection and many plants died after 40 days. The most important result of this research is that disease develops slower in plants up to 36 days old at the time of inoculation, but then appears to develop at a faster rate for plants older than 36 days. Plants may be susceptible at all stages of growth, but some resistant mechanism seems to lower the rate of disease development up to a certain plant age. These results do not seem to agree with field observations the last few years that older plants do not become infected under ideal conditions for infection. The results also may

be related to the availability of infection sites, the regions of the roots between the root apex and the root hair zone (Mehrotra, 1969) where Phytophthora megasperma f. sp. glycinea infects initially. In the greenhouse experiment these root areas were always accessible to infection by zoospores (Fig 12), whereas in the field these root areas may be too deep in the soil profile to become infected by zoospores. It may be that the older plants grown in large containers became more stressed as they grew.

There was a significant reduction ($P=0.05$) in the fresh weight of roots from inoculated plants in the growth chamber experiment (Table 2). This agrees with Meyer et al (1972), who found that Phytophthora megasperma var. sojae reduced the root system of soybeans whether stem lesions were present or not. The reduction in foliar dry weight seems to be more important in older plants: the reduction in plants inoculated at 21 days of age was 45.8 % of the non-inoculated control (growth chamber experiment), and 60.3% in plants inoculated at 50 days of age (greenhouse experiment). The number of surviving plants 40 days after inoculation increased as plant age increased up to 36 days at the time of inoculation. (Fig 10). These results confirm that Phytophthora megasperma f. sp. glycinea can attack soybean plants at any stage of growth, but that up to flowering stage some form of resistance slows down the rate of disease development. Fewer plants inoculated at 43 and 50 days of age survived, suggesting again that older

plants have less resistance to disease development. As discussed earlier, this contradicts field observations and could be related to the availability of infection sites. Other explanations are possible, including reduced inoculum potential, antagonisms or other types of soil suppressiveness associated with mid-summer soil conditions. Suppressiveness has been reported for other root rot diseases, especially the *Fusarium* wilts (Lockwood, 1977), but seasonal suppressiveness has not been reported. Antagonists of *P. m. glycinea* have also been reported (Malajczuk, 1983) and may be important under some conditions. These areas need to be investigated.

The statistical method of evaluation, a two-way analysis of variance and Duncan's Multiple Range test were valid analysis because the plants of different ages were inoculated at the same time. If the plants had been inoculated at different times, a factorial analysis would have been appropriate.

Disease evaluation based on the disease rating and number of surviving plants was the most relevant comparison among plants of different ages because the statistical analysis for dry weight of foliage and fresh weight of roots was based on means derived from as few as one plant to as many as 25.

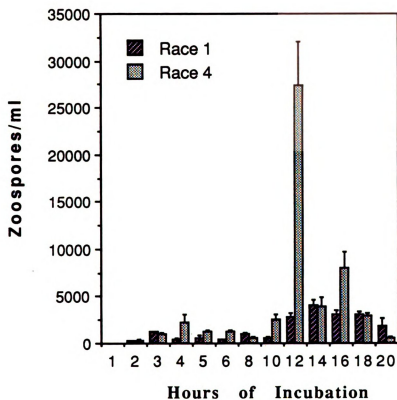


Figure 1. Production of zoospores by races 1 and 4 of *Phytophthora megasperma* f. sp. *glycinea*. Each treatment was replicated three times and six counts with a hemacytometer were made per treatment.

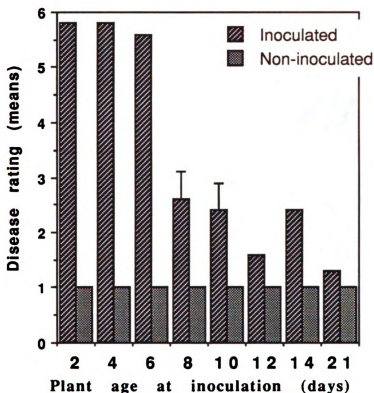


Figure 2. Disease rating of inoculated and non inoculated plants of the cultivar Conrad, 8 days after inoculation with a zoospore suspension (2.7×10^4 /ml) of *P. m. glycinea*, race 4. Each value is the average of 25 (inoculated) and 5 (non-inoculated) plants, from the growth chamber experiment. (The zero and seven values of disease rating was put in for display purposes. The lowest and highest ratings were always one and six).

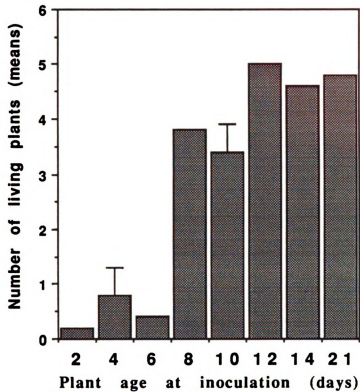


Figure 3. Number of plants of the cultivar Conrad remaining alive 20 days after inoculation with a zoospore suspension (2.7×10^6 /ml) of Phytophthora megasperma f. sp. glycinea race 4 in the growth chamber experiment.

Table 1. Two- way analysis of variance for disease rating and the percentage of plants living at the end of the experiment for plants inoculated at different ages. The cultivar Conrad was inoculated with a zoospore suspension (2.7×10^4 /ml of Phytophthora megasperma f. sp. glycinea race 4.

Plant age at inoculation (days)	Disease rating	Plant survival 20 days after inoculation (%)
21 days	1.3 a	88.0 a b
12 days	1.6 a b	100.0 a
14 days	2.4 b	92.0 a b
10 days	2.4 b	68.0 b
8 days	2.6 b	76.0 a b
6 days	5.6 c	8.0 c
4 days	5.8 c	16.0 c
2 days	5.8 c	4.0 c
F (Treatments)	32.96 **	21.28 **
F (Block or tray)	0.32 NS	0.10 NS
CV. (%)	22.2	34.6
Test:	Duncan	Duncan

Means followed by the same letter are not significantly different (Duncan's Multiple Range test $P = 0.05$)

** Differences highly significant ($p < 0.01$)

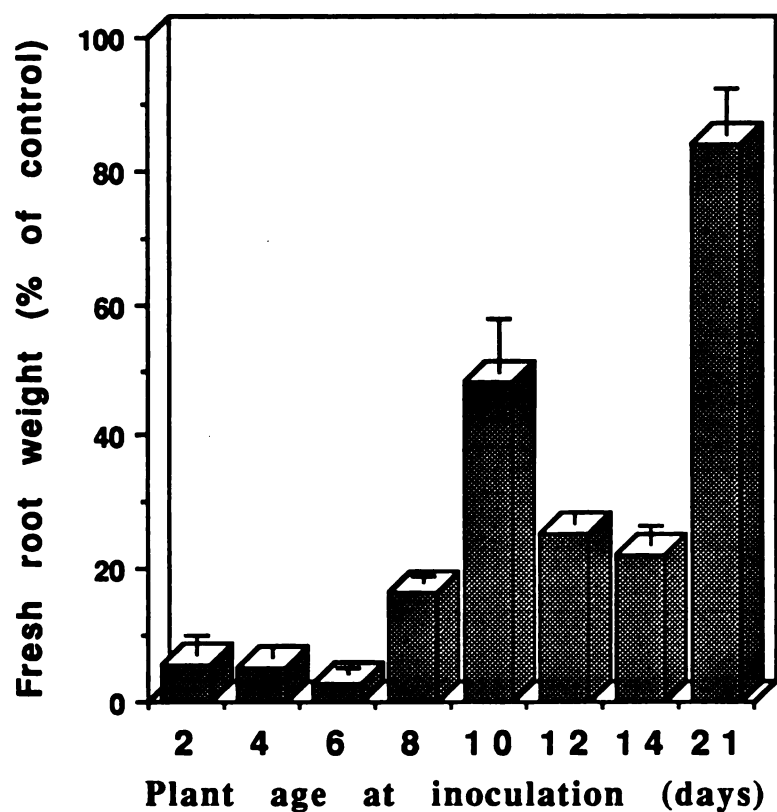


Figure 4. Fresh root weight (g) of inoculated plants (Av. of 25 plants) as a percentage of control plants fresh root weight (Av. of 5 plants) of the cultivar Conrad. Plants were inoculated with a zoospore suspension (2.7×10^6 /ml) of Phytophthora megasperma f. sp. glycinea race 4 in the growth chamber experiment.

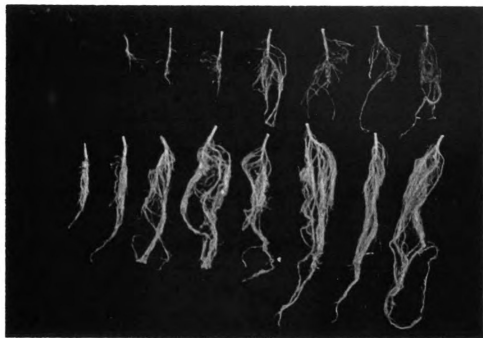


Figure 5. General root rot of primary and secondary roots of inoculated plants (top) of the cultivar Conrad in the growth chamber experiment. From the left to the right roots of plants inoculated 4, 6, 8, 10, 12, 14 and 21 days of age, and harvested 30 days after inoculation. Roots of 2-day-old inoculated plants were completely rotted. Lower roots are from non-inoculated plants.

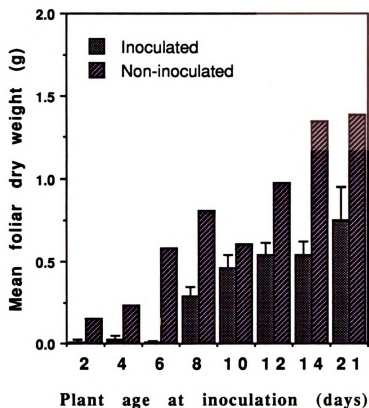


Figure 6. Foliar dry weight (g) of inoculated (Av. of 25 plants) and non-inoculated (Av. of 5) plants of the cultivar Conrad from the growth chamber experiment. Inoculation was made with a suspension of zoospores (2.7×10^4 /ml) of Phytophthora megasperma f. sp. glycinea race 4.

Table 2. Two- way analysis of variance for fresh root weight (% of the control) and foliar dry weight (% of the control) at different plant ages when inoculated the cultivar Conrad with a zoospore suspension (2.7×10^4 /ml) of *P. m. glycinea* race 4 in the growth chamber experiment.

Plant age at inoculation (days)	Fresh root weight (% of control)	Foliar dry weight (% of control)
21 days	84.2 a	54.3 ab
10 days	48.2 b	77.3 a
12 days	25.5 c	55.5 ab
14 days	21.9 c	39.9 b
8 days	16.6 c	36.5 b
2 days	5.6 c	6.7 c
4 days	5.3 c	11.8 c
6 days	2.7 c	1.7 c
F (treatments)	14.3 **	10.9 **
F (blocks or trays)	1.30 NS	0.71 NS
CV. (%)	62.8	51.2
Test:	Duncan	Duncan

Means followed by the same letter are not significantly different (Duncan's Multiple Range test $P=0.05$)

** Differences highly significant ($P < 0.01$).

NS Differences no significant.

Means to determine weight as a percent of the control plants were calculated from plants living 30 days after inoculation (see Table 1).

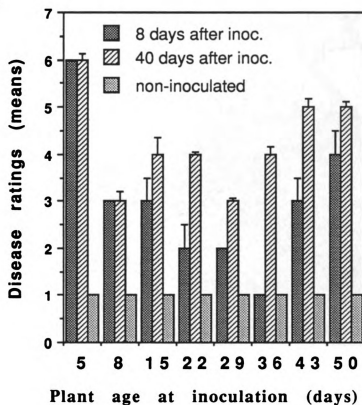


Figure 7. Disease ratings of inoculated and non-inoculated plants of the cultivar Sloan, 8 and 40 days after inoculation with a zoospore suspension (7.1×10^3 /ml) of *Phytophthora megasperma* f. sp. *glycinea* race 4. (The zero and seven values of disease ratings were put in for display purposes. The lowest and highest rating were always one and six).



Figure 8. Soybean plants of the susceptible cultivar Sloan, one week after inoculation with a zoospore suspension (7.1×10^5 /ml) of *P. m. glycinea* race 4 in the greenhouse experiment. See the wilting and yellowing specially in older plants.



Figure 9. Left: Soybean plants of the susceptible cultivar Sloan, 40 days after inoculation with a zoospore suspension (7.1×10^6 /ml) of *P. m. glycinea* race 4. Right: Non inoculated plants.

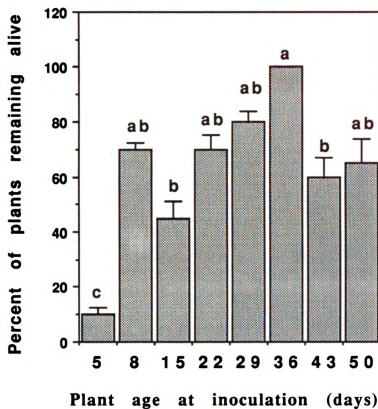


Figure 10. Percent of plants of the cultivar Sloan remaining alive 40 days after inoculation with a zoospore suspension (7.1×10^6 /ml) of *P. m. glycinea* race 4. Means followed by the same letter are not significantly different at $P = 0.05$ (Duncan's multiple range test).

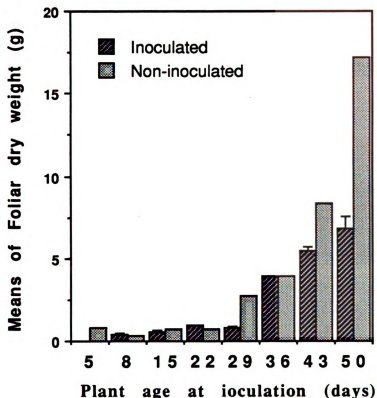


Figure 11. Foliar dry weight of inoculated and non inoculated plants of the cultivar Sloan harvested 40 days after inoculation with a zoospore suspension (7.1×10^6 / ml) of Phytophthora megasperma f. sp. glycinea race 4 in the greenhouse experiment. Means to determine weight as a percent of the control plants were calculated from plants living 40 days after inoculation.

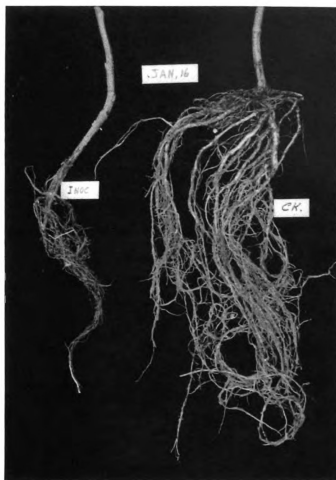


Figure 12. Left: General root rot of primary and secondary roots of a soybean plant of the cultivar Sloan inoculated when plants were 50 days old with a zoospore suspension (7.1×10^7 /ml) of *P. m. glycinea* race 4 and harvested 40 days later. Right: Root of one non-inoculated plant.

CHAPTER 2.

CHAPTER 2

Role of alternate hosts on the survival of Phytophthora megasperma f. sp. glycinea.

INTRODUCTION AND LITERATURE REVIEW

Isolates of Phytophthora megasperma f. sp. glycinea (= Phytophthora megasperma Drechsler var. sojae) that are pathogens of hosts other than soybeans have been described. Van der Zwet (1961), described a Phytophthora on seed-pieces of sugarcane in Louisiana, that was almost identical to isolates of Phytophthora described by Hildebrand in 1959, that were highly specific for soybeans. Jones et al. (1969), reported the isolation of Phytophthora megasperma var. sojae from diseased seedlings of the cultivated white, blue and yellow lupines (Lupinus albus, L. angustifolius and L. luteus respectively) grown in naturally infested soils. Three wild species, L. bicolor, L. densiflorus and L. succulentus, also proved to be susceptible to the soybean fungus Phytophthora megasperma var. sojae. All six species are considered hosts of the pathogen. He found that Phytophthora megasperma var. sojae was not simultaneously introduced to North America with soybeans, but was endemic on native lupines.

Boesewinkel (1974), reported Phytophthora megasperma var.

sojae for the first time in asparagus in New Zealand. It caused death in commercial and noncommercial crops and was present in spears offered for sale. Asparagus spears, apples (Malus sylvestris) and tomatoes (Lycopersicon esculentum) artificially inoculated by placing the fungus under the skin, were readily infected with abundant formation of oospores in the rotting tissues. Stem puncture inoculation of 3-week old seedlings killed 100% of the asparagus seedlings, 80 % of blue lupin (Lupinus angustifolius) and 44 % of 50 tomato seedlings. Irwin, 1974, reported that the causal organism of root rot of lucerne (*Medicago sativa* L.) was identified as Phytophthora megasperma Drechsler var. sojae Hildebrand. The disease caused serious reductions in lucerne productivity in many areas of Queensland (Irwin, 1976). He reported that zoospores penetrated the roots of lucerne in solution cultures. Singh et al, (1979), described a destructive root rot disease of cauliflower and cabbage in Varnasi, and identified the pathogen as Phytophthora megasperma var. sojae according to morphology, developmental and cultural characteristics. In another publication (1979), he describes the mode of infection and development of the pathogen in cauliflower roots.

Vock et al, (1980), isolated Phytophthora megasperma var. sojae from a severe root rot of a commercial crop of Cicer arietinum cv. Tyson in July 1979 and reproduced the symptoms in inoculation tests. Subsequently, Phytophthora megasperma var. sojae was isolated from chickpeas with similar symptoms

at four other locations in Queensland. This appear to be the first record of Phytophthora megasperma var. sojae infecting chickpea where the fungus has been positively identified and pathogenicity tests conducted. Chevis et al, (1982), in Australia, studied the scattered sudden death of young Pinus radiata. Transplanting losses were generally high, and mortalities gradually declined in the early years after planting, with death ceasing in unthinned stands by age 7 to 8 years. Phytophthora spp. were associated with this problem and P. megasperma var. sojae was isolated from rotting fine roots of two apparently healthy 1-year old Pinus radiata plants. Fallon et al, (1988), isolated P. megasperma var. sojae from asparagus plants and soils in California. This pathogen was previously reported in asparagus, in Australia (McGeary et al, 1985), New Zealand (Boesewinkel, 1974) and Switzerland (Gindrat, personal communication).

Ta-Li Kuan et al. (1980), reported that isolates of Phytophthora megasperma from alfalfa and soybean were pathogenic only to the hosts from which they were isolated and proposed special forms to subdivide the alfalfa and soybean isolates of P. megasperma, into P. megasperma f. sp. medicaginis for isolates that attack alfalfa and P. megasperma f. sp. glycinea for isolates that infect soybean. This was the first attempt to separate isolates of Phytophthora megasperma based on host specificity and consequently, created a question about the previous reports of P. megasperma var.

soiae as pathogens of many hosts. Hamm et al. (1981), also found that isolates of P. megasperma from alfalfa and soybean were pathogenic only to their original hosts, but one group of isolates from Douglas fir was strongly pathogenic to both Douglas fir and soybean. This group was virulent on seven soybean cultivars used to define races of Phytophthora megasperma f. sp. glycinea, and on the cultivar Tracy (Hamm et al., 1981). This indicated that some plant species may be common hosts for populations of P. megasperma which differ in pathogenic specialization, or that host ranges of different formae speciales may overlap. Therefore, some forma speciales of P. megasperma, have a wider host range including soybeans, but for P. megasperma f. sp. glycinea isolated from soybean, the host range is apparently limited to soybean.

Irwin et al. (1982), investigated the relationship between morphologically indistinguishable P. megasperma isolates from soybean (P. megasperma f. sp. glycinea), lucerne (P. megasperma f. sp. medicaginis) and chickpea, using the criteria of growth temperature, specific pathogenicity, capacity to cross protect and electrophoretic patterns of buffer soluble proteins. Their results showed that isolates from soybean could readily be distinguished from the chickpea and lucerne isolates, but the chickpea and lucerne isolates could not be distinguished. These results agreed with the designation of formae specialis by Ta-Li Kuan et al, 1980. Wilcox et al, (1987), found that isolates of P. megasperma

from soybean, caused moderate root rot, a 40 - 62 % decrease in root weight and a 37 - 75 % decrease in shoot weight on Mahaleb cherry seedlings relative to uninoculated control, but no crown rot or plant death. They also reported that these isolates were weakly to moderately virulent on alfalfa.

The extent of pathogenic specialization within *P. megasperma* is, therefore, an important consideration from a disease management perspective. For instance, common strategies designed to control disease through the reduction or exclusion of inoculum (e.g., sanitation or crop rotation) are fundamentally dependent on knowledge of what potentially constitutes inoculum. That is, the potential for a particular isolate of *P. megasperma* to cause damaging levels of disease on more than one crop can directly influence the development of disease management practices for the crop in question.

Based on these studies, rotation of soybeans with other crops including wheat, alfalfa, etc, was not considered a problem. Inoculum levels should not increase on this hosts. However, none of this studies have considered the possible survival of *P. megasperma* f. sp. *glycinea*, in alternate or non hosts, in which it is not pathogenic but where zoospores could infect and subsequently produce oospores in the roots. These alternate hosts could be a source of infection for next susceptible soybean crop.

Ho (1969), found that root extracts and roots of a highly resistant (Harosoy 63) and a highly susceptible (Harosoy)

soybean var. and of a nonhost plant (Alaska pea), all stimulated the growth of P. megasperma var. sojae but suppressed sporangia formation. Effects were non specific as regards resistance and susceptibility, host and nonhost. No evidence for formation of oospores in the roots was presented.

Mehrotra (1970), found that in general zoospores of P. dreschleri and P. megasperma var. sojae accumulated both on host (resistant and susceptible cultivars) and non host plant roots in soil, indicating the accumulation or attraction phenomenon to be non specific. In studies of root infecting Phycomycetes, several investigators have drawn attention to the massing of zoospores on roots in vitro especially just behind the root tip. One question that arises is what is the significance of zoospore accumulation in disease production.

Irwin, (1976), indicated that zoospores of P. megasperma var. sojae, exhibited a similar tactic response and penetration of roots of both the resistant cultivar Lahontan and the susceptible cultivars Hunte, River and Moapa of lucerne.

In this chapter, studies about behavior of Phytophthora megasperma f. sp. glycinea in alternate hosts and the possible importance of these alternate hosts in disease production are presented.

MATERIALS AND METHODS

Resistant and susceptible soybean (cultivars Dassel and Sloan), wheat (var. Frankenmuth), alfalfa (var. Iroquois), bean (var. Black Magic) and corn were inoculated with *P. megasperma* f. sp. *glycinea* race 4 and examined for oospore production within the roots in two greenhouse experiments, one in vermiculite and other in steamed soil.

Preparation of plants.

All the seeds were surface sterilized with 0.525% NaOCl and two drops of tween 20, for 20 minutes, washed three times with sterilized distilled water, and planted in vermiculite and steamed soil in 30 cm long, 25 cm wide and 5 cm high aluminum trays. Seventy seeds were planted in each tray.

Preparation of inoculum.

Inoculum was prepared using cultures of *Phytophthora megasperma* f. sp. *glycinea* race 4 on V-8 juice agar, incubated in the dark at 24°C for 12 days. To get maximum production of zoospores, the cultures were washed four times (20 min each time) with salt solution (10% Aphanomyces Replacement Solution), flooded with sterilized distilled water and incubated overnight in the dark at 24°C. After 12-16 hours of incubation zoospores were collected and counted using the microsyringe method (Ko et al, 1973).

Inoculation

1. Inoculation in vermiculite.

a. Before planting, V-8 agar cultures of *P. megasperma* f. sp. *glycinea* race 4, 12 days old were placed on the surface of vermiculite and covered with a 3 cm layer of vermiculite. Six culture plates of the fungus were used on each tray 30 cm long, 25 cm wide and 5 cm high.

b. After planting, plants three days old were inoculated with 208 ml per tray of a zoospore suspension of 1.3×10^3 /ml of *P. megasperma* f. sp. *glycinea* race 4.

2. Inoculations in steamed soil.

a. Before planting, V-8 agar cultures of *P. megasperma* f. sp. *glycinea* race 4, 12 days old were placed on the surface of the steamed soil and covered with a 3 cm layer of steamed soil. Six culture plates of the fungus were used on each tray.

b. After planting, plants three days old were inoculated with 236 ml per tray of a suspension of $6,2 \times 10^3$ zoospores/ml of *P. m. glycinea*. race 4.

Each of the above treatments was made in one tray for each plant species tested. After inoculation all the trays were flooded with water for three days.

Sampling

A sample of ten plants per tray was taken 5 days after inoculation to analyze for the presence of oospores in the

roots. Five root systems of each sample were stained with Chloral hydrate-acid fuchsin clearing-staining solution (830 g of chloral hydrate, 100 ml of water and 0.1 g of acid fuchsin) and five were used to reisolate the fungus.

Observation of the stained roots.

To stain oospores, the roots were put into boiling staining solution for 20 minutes, washed once with distilled water and put in beakers sealed with parafilm to maintain humid condition. To observe under the microscope, the root system of each plant was sectioned when necessary, crushed between two slides, embedded in lactophenol (20 ml of Phenol warmed to melt, 20 ml of lactic acid, 40 ml of glycerin and 20 ml of distilled water) and covered with cover glasses.

Reisolation of the fungus from infected roots.

Two techniques were used: a) The roots were cut into small pieces and put directly onto Phytophthora selective medium. b) Baiting technique: the roots were surface sterilized with 70% alcohol, washed three times with sterilized distilled water and maintained flooded in sterilized water for two days, at room temperature in a 200 ml beaker. After that, 24 discs 5 mm in diam of susceptible soybean leaves from plants less than 20 days old, were added per beaker. Beakers were kept in the dark at room temperature. The discs were observed under the microscope, two hours, 1 day, 2 days and 4 days later. If

sporangia were observed, the discs were transferred to Phytophthora selective medium. Then, the Phytophthora colonies would be transferred to clarified V-8 juice agar (200 ml of V-8 juice, 2 gr of CaCO₃ (heat V-8 juice and Calcium carbonate to 80°C and after cooling, centrifuge in large rotor at 9000 RPM for 10 min), 20 g of agar and 800 ml of water).

Phytophthora selective medium.

Different selective media were tried in order to avoid Pythium contamination:

1. (Schmitthenner) 40 ml of V-8 juice neutralized by 2 g of Ca CO₃ (2 g CaCO₃/ 1 of V-8 juice. Heat at 80C and centrifuge to clarify), 0.2 g of yeast extract, 1.0 g sucrose, 0.025 g of 75% PCNB, 0.02g of 50 % Benomyl, 0.01 g of Chloranfenicol, 0.1 g of Neomycin, 20 g agar and 960 ml of water.
2. Jeffers et al, (1986). Autoclave 1 l Difco corn meal agar (17 g/l) without glucose and cool to 50°C. Add 5 mg pimarinic, 250 mg ampicillin, 10 mg rifampicin, 100 mg PCNB and 5 mg Bemomyl.
3. Bielenin et al, (1988). To one liter of water, add 20 ml of clarified V-8 juice, adjust the pH to 6.5 before autoclaving and add 17 g of agar. Add 5 mg of Benomyl, 5 mg of hymexazol, 5 mg of pimarinic and 25 mg of rifampicin after autoclaving and cooling.

Infection of susceptible soybeans planted in soil infested with roots of non-hosts plants previously infested by *P. megasperma* f. sp. *glycinea*

Soybean, wheat, dry bean and alfalfa seeds were surface sterilized with 0.525% NaOCl and two drops of Tween 20 for 20 minutes, washed three times in sterilized distilled water and planted in steamed soil (the soil was steamed in trays for 30 minutes by autoclaving at 120°C). Each tray contained a 5 cm thick layer of soil (about 2,120 g of soil /tray). Plants three days old were inoculated with 367 ml per tray of a suspension of 1×10^3 zoospores/ml. After inoculation the trays were flooded with water for three days, let dry and flooded again. Each treatment was replicated three times. A control of inoculated soil without plants, and a control of non-inoculated soil was also included. Ten days after inoculation, all the plants were cut, leaving the roots in the soil (The roots should have been examined for infection, that is, presence of oospores). The trays were let dry for ten more days. After this time, 25 seedlings of the soybean var. Sloan (germinated in humid paper towels) were planted in each tray. All the trays were flooded with water for three days, let dry and flooded again several times. The plants that remained alive 20 days after planting were counted and the roots examined for the presence of rot symptoms, dark discoloration and oospores. Controls included steamed soil

infected with *P. m. glycinea* as on the other treatments, but no plants planted. This was to test for survival of *P. m. glycinea* and later infection in the absence of a host in which to form oospores.

RESULTS

Presence of oospores in stained roots observed under the microscope.

The results of each treatment are shown in the Tables 3 to 8 and Figs 13 to 23. Roots of plants grown in vermiculite and collected 7 days after inoculation with *Phytophthora megasperma* f. sp. *glycinea* in general showed higher number of oospores when inoculated with the colony of the fungus (oospores and mycelium) in the soil, as compared to zoospore inoculum, except for the susceptible soybean cultivar Sloan (Tables 3 and 4). Roots of the dry bean cultivar Black magic had the highest numbers of oospores among the alternate hosts and the resistant soybean cultivar Dassel. Roots of wheat had different number of oospores in all the roots analyzed when inoculated with the colony of the fungus in vermiculite (Table 3) but had no oospores when inoculated with the zoospore suspension (Table 4). Alfalfa roots had a few oospores when inoculated with the colony of the fungus deep in vermiculite (Table 3) but also had no oospores when

inoculated with the zoospore suspension (Table 4). Stained roots of the resistant variety Dassel showed some oospores regardless of the inoculum. Corn roots had only two oospores in one plant, when inoculated with the colony of the fungus in vermiculite (Table 3) and was not included in the next experiment in steamed soil.

The number of oospores on each root of plants grown in steamed soil and inoculated with the colony of the fungus (Tables 5 and 6) was higher and more consistent in plants collected 8 days after flooding (Table 6). It appeared that roots of the susceptible soybean Sloan and dry beans were more quickly colonized than roots of wheat, alfalfa and the resistant soybean Dassel (Tables 5 and 6 and Figs 18 and 21). The number of oospores observed in roots of all plant species inoculated with a zoospore suspension in steamed soil (Tables 7 and 8) was higher in plants collected eight days after inoculation, (Table 8) than three days after inoculation (Table 7). This occurred with roots of the soybean cultivar Sloan, dry beans and alfalfa. In wheat roots there were no differences, and in the soybean cultivar Dassel there was more oospores in the roots three days after inoculation.

Reisolation of the pathogen from colonized roots.

The pathogen was baited onto leaf discs of soybeans from roots of the soybean cultivar Sloan, the resistant soybean cultivar Dassel and the dry bean cultivar Black magic, four days after

incubation of flooded infected roots. Sporangia could be seen arising from the border of the leaf discs (Fig 24). Leaf discs with the sporangia were transferred to Phytophthora selective medium, but the fungus could not be reisolated due to contamination with Pythium. According with Dr. Schmitthenner (personal communication), susceptible soybean seedlings should be used instead of leaf discs, and shorter periods of incubation, in order to avoid Pythium contamination. The pathogen was not baited from infected roots of wheat and alfalfa.

Table 3. Number of oospores of Phytophthora megasperma f. sp. glycinea race 4 in roots of plants grown in vermiculite and collected seven days after inoculation with a colony of the fungus as a layer 3 cm deep in the soil.

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	1000	>1000	>1000	500	1000	0.0
Soybean (Dassel)	0.0	0.0	1.0	600	0.0	0.0
Wheat	105	252	4	20	123	0.0
Alfalfa	7	32	6	17	0.0	0.0
Dry Bean	500	350	1000	500	500	0.0
Corn	00	2	0.0	0.0	0.0	0.0

* Ck is the average of 5 plants.

Table 4. Number of oospores of P. m. glycinea race 4 in stained roots of plants grown in vermiculite and collected seven days after inoculation with a zoospore suspension (1.3×10^5 /ml).

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	>1000	>1000	>1000	>1000	>1000	0.0
Soybean (Dassel)	0.0	0.0	0.0	114	0.0	0.0
Wheat	0.0	0.0	0.0	0.0	0.0	0.0
Alfalfa	0.0	0.0	0.0	0.0	0.0	0.0
Dry Bean	62	2	600	160	250	0.0
Corn	0.0	0.0	0.0	0.0	0.0	0.0

* Ck. is the average of 5 non inoculated plants.

Table 5. Number of oospores of P. megasperma f. sp. glycinea race 4, in stained roots of plants grown in steamed soil and collected three days after inoculation with a colony of the fungus as a layer 3 cm deep in the soil.

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	413	800	1500	39	49	0.0
Soybean (Dassel)	-	-	-	-	-	0.0
Wheat	0.0	0.0	0.0	0.0	0.0	0.0
Alfalfa	0.0	0.0	0.0	0.0	0.0	0.0
Dry Bean	1400	150	400	384	350	0.0

* Ck. is the average of 5 non inoculated plants.

Table 6. Number of oospores of P. megasperma f. sp. glycinea race 4 in roots of plants grown in steamed soil and collected eight days after inoculation with a colony of the fungus as a layer 3 cm deep in the soil.

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	2300	1000	1000	500	400	0.0
Soybean (Dassel)	0.0	400	0.0	450	150	0.0
Wheat	221	500	140	107	100	0.0
Alfalfa	113	0.0	0.0	0.0	0.0	0.0
Dry Bean	1650	100	430	450	1350	0.0

* Ck is the average of 5 non inoculated plants.

Table 7. Number of oospores of P. megasperma f. sp. glycinea race 4 in stained roots of plants grown in steamed soil and collected three days after inoculation with a zoospore suspension (6.2×10^3 /ml).

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	500	400	300	500	-	0.0
Soybean (Dassel)	1200	450	1000	800	59	0.0
Wheat	0.0	0.0	0.0	0.0	0.0	0.0
Alfalfa	0.0	200	0.0	0.0	0.0	0.0
Dry Bean	400	650	250	215	270	0.0

* Ck. is the average of 5 non inoculated plants.

Table 8. Number of oospores of P. megasperma f. sp. glycinea race 4 in stained roots of plants grown in steamed soil and collected eight days after inoculation with a zoospore suspension (6.2×10^3 /ml).

Roots	1	2	3	4	5	Ck.*
Soybean (Sloan)	2500	2000	1000	1000	1000	0.0
Soybean (Dassel)	460	700	8	150	-	0.0
Wheat	0.0	0.0	0.0	0.0	2	0.0
Alfalfa	62	0.0	105	13	0.0	0.0
Dry Bean	1000	750	700	550	1540	0.0

* Ck. is the average of 5 non inoculated plants.

DISCUSSION

Phytophthora megasperma f. sp. glycinea colonized roots of non-hosts plants, as well as resistant and susceptible cultivars of soybeans. These results agree with Ho, (1969), Mehrotra, (1970) and Irwin (1976) in that roots of host and non-hosts plants can attract zoospores, and expands that work by showing the formation of oospores in the roots (Figs 17 to 23). This strongly suggests that symptomless non-hosts of P. m. glycinea can serve as sources of inoculum and as infection sites for P. m. glycinea zoospores. Inoculation with a colony of the fungus (oospores and mycelium) as a layer deep in vermiculite or soil probably resulted in much greater zoospore production and may explain why in general higher levels of infection were observed. As might be expected, the susceptible soybean cultivar Sloan supported higher number of oospores than any of the other hosts. However, dry beans and often the resistant cultivar Dassel also had high number of oospores in some experiments, especially in steamed soil as compared to vermiculite. Discoloration of the inoculated roots occurred both in soybean cultivar Sloan and dry beans (Figs 14 and 15). Wheat and alfalfa (Fig 16) had a few discolored roots when planted in steamed soil. Roots of plants collected eight days after flooding had higher number of oospores, which may be due to better conditions for zoospore-movement.

The conclusions are that roots of non-host plants can attract zoospores of *P. megasperma* f. sp. *glycinea* and that these zoospores can encyst and infect the roots with later formation of oospores. Therefore, the presence of oospores in non-host roots can be a potential source of infection for the next susceptible soybean crop. The high level of infection found in dry beans suggest that it may be an important symptomless host for *P. megasperma* f. sp. *glycinea* and future rotational recommendations may have to take these results into account.

Although the experiments using whole cultures of *P. m. glycinea* as inoculum may appear to be unnatural, there may be field situations where high levels of oospores are aggregated in decaying root/stem debris and high levels of sporangia and zoospores produced. The role of mycelial infection of roots under artificial conditions similar to those described here have not been reported. However, hypocotyl inoculations with mycelium of *P. m. glycinea* is a common method of screening for varietal resistance and race determination.



Figure 13. Bottom: Soybean plants of the cultivar Sloan planted in vermiculite one week after inoculation. Left to the right: Plants inoculated with a colony of the fungus deep in the vermiculite, non-inoculated plants and plants inoculated with a zoospore suspension of *P. m. glycinea* race 4. Top: The resistant cultivar Dassel.



Figure 14. General root rot of susceptible soybean plants (cultivar Sloan) planted in steamed soil and inoculated with colony of *P. m. glycinea* deep in the soil (left) and a zoospore suspension (right). Roots in the center are from non-inoculated plants.



Figure 15. General root rot of primary and secondary roots of the non-host bean plants (cultivar Black magic) planted in steamed soil and inoculated with a colony of *P. m. glycinea* deep in the soil (left) and a zoospore suspension (right). Roots in the center are from non-inoculated plants.

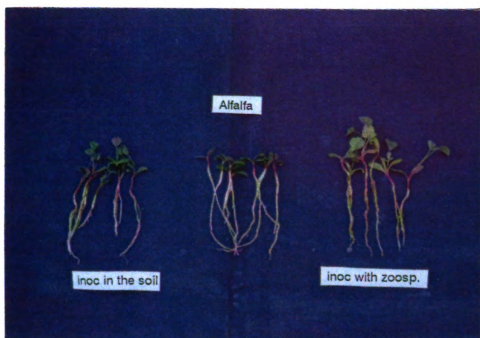


Figure 16. Slight brown discoloration of roots of the non-host alfalfa planted in steamed soil and inoculated with the colony of *P. m. glycinea* deep in the soil (left) and a zoospore suspension (right). Roots in the center are from non-inoculated plants.

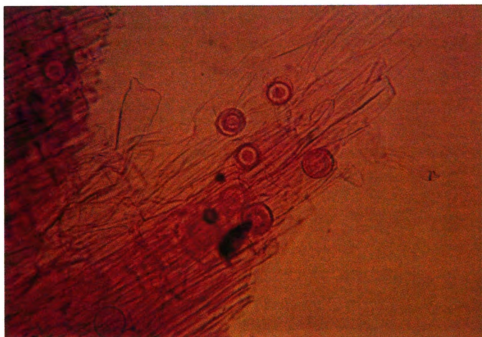


Figure 17. Oospores formed in infected root tissues of susceptible soybean plants, stained with chloral hydrate-acid fuchsin clearing- staining solution (magnification 200 X).

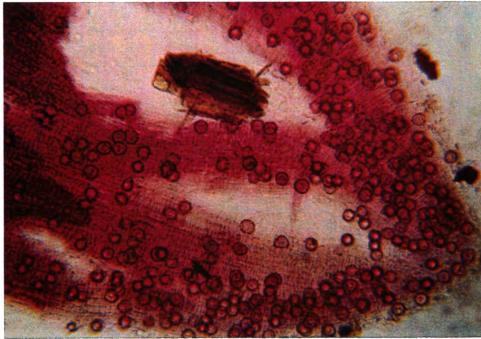


Figure 18. Root tip of primary root of susceptible soybean (cultivar Sloan) inoculated with *P. m. glycinea*. Oospores are evenly distributed in all the roots. Roots were stained with chloral hydrate-acid fuchsin clearing-staining solution. (Magnification: 100 X)

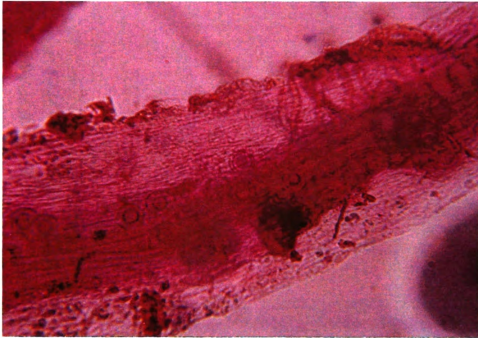


Figure 19. Oospores inside a lateral root of the resistant soybean cultivar Dassel, stained with chloral hydrate-acid fuchsin clearing staining solution. (Magnification: 200 X)

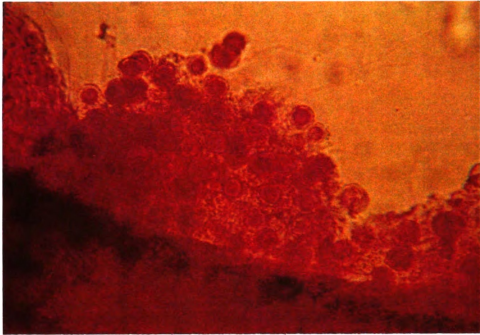


Figure 20. Oospores accumulated outside of a lateral root of the resistant soybean cultivar Dassel, when inoculated in the soil with a colony of the *P. m. glycinea*. (Magnification: 200 X).

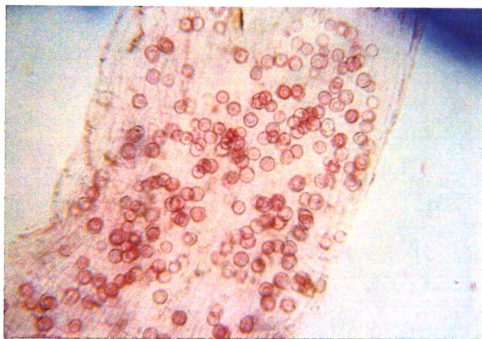


Figure 21. Oospores formed inside of the root tissues of the inoculated non-host bean plant cultivar Black magic. (Magnification: 100 X).

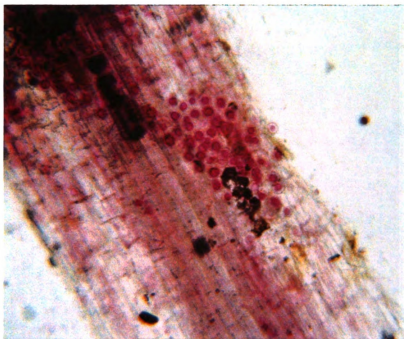


Figure 22. Oospores formed in inoculated roots of the non-host plant wheat cultivar Frankenmuth.
(Magnification: 100 X)

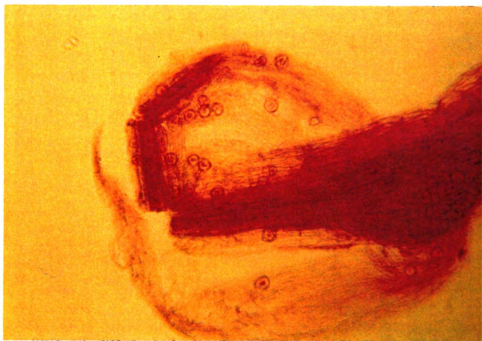


Figure 23. Oospores formed in inoculated roots of the non-host plant alfalfa cultivar Iroquois. (Magnification: 100 X).



Figure 24. Sporangium formed on the border of susceptible soybean leaf discs, when a baiting technique was used to reisolate P. m. glycinea from infected roots of host and non-host plants. (Magnification: 100 X)

APPENDIX

APPENDIX

Table A 1. Two-way analysis of variance for disease ratings 8 and 40 days after inoculation of plants of the cultivar Sloan with a zoospore suspension (7.1×10^3 /ml) of *P. m. glycinea* race 4 in the greenhouse experiment.

Age at inoculation (days)	Disease rating 8 days after inoc.		Disease rating 40 days after inoc.	
5 days	5.5	a	5.6	a
50 days	3.8	b	4.8	a b
15 days	3.5	b	3.9	b c
8 days	3.0	b	3.0	c
43 days	2.8	b c	5.0	a b
22 days	2.3	b c	3.8	b c
29 days	2.0	b c	3.3	c
36 days	1.0	c	3.5	c
F (Treatments)	5.8	**	5.21	**
F (Blocks)	0.03	NS	0.49	NS
CV. (%)	37.6		19.9	
Test.	Duncan		Duncan	

Means followed by the same letter are not significantly different (Duncan's Multiple Range test $P=0.05$).

** Differences highly significant ($P < 0.01$).

NS Differences no significant.

Table A 2. Two-way analysis of variance for foliar dry weight of inoculated plants as a percent of the non-inoculated plants. Cultivars Sloan (S) and Dassel (R) were inoculated with zoospore suspension (7.1×10^3 /ml) of *P. m. glycinea* race 4 in the greenhouse experiment.

Plant age at inoculation (days)	Dry weight of aerial parts of the plants					
	Var: Sloan			Var: Dassel		
	Inoc. (%)	Ck. (g)		Inoc. (%)	Ck. (g)	
8 days	150 a	0.3		83.7 a	0.8	
22 days	135 a	0.7		64.8 a	1.1	
36 days	100 ab	3.9		71.5 a	3.7	
15 days	71.4 bc	0.8		152.6 a	0.5	
43 days	66 bc	8.4		90.6 a	9.4	
50 days	39.7 cd	17.2		109.1 a	8.6	
29 days	29.3 cd	2.7		121.6 a	0.9	
5 days	0.00 d	0.8		116.0 a	0.5	
F	11.5 **			2.4 NS		
CV. (%)	29.4			37.0		
Test:	Duncan			Duncan		

Means followed by the same letter are not significantly different (Duncan's Multiple Range test $P=0.05$)

Table A 3. Disease rating of inoculated (I,II,II,IV and V) and non inoculated (Ck.) plants of the cultivar Conrad, 8 days after inoculation with a zoospore suspension (2.7×10^4 /ml) of Phytophthora megasperma f. sp. glycinea race 4. Each number is the average of five plants.

Plant age at inoculation	I	II	III	IV	V	Av.	Check
2 days	6	5	6	6	6	5.8	1
4 days	6	6	6	6	5	5.8	1
6 days	5	6	5	6	6	5.6	1
8 days	3	3	1	2	4	2.6	1
10 days	2	4	3	2	1	2.4	1
12 days	2	1	2	2	1	1.6	1
14 days	3	2	3	2	2	2.4	1
21 days	1	-	1	1	1	1.3	1

Table A 4. Fresh root weight (g) of plants of the cultivar Conrad inoculated with a zoospore suspension (2.7×10^6 /ml of *P. m. glycinea*. race 4. Data are the average of five plants. (*))

Age at inoculation	I	II	III	IV	V	Av.	Ck.
2 days	0.00	0.1	0.00	0.00	0.00	0.02	0.36
4 days	0.08	0.08	0.00	0.00	0.00	0.03	0.6
6 days	0.06	0.00	0.14	0.00	0.00	0.04	1.48
8 days	0.48	0.54	0.62	0.32	0.52	0.5	2.98
10 days	0.72	0.36	0.13	0.61	1.36	0.63	1.32
12 days	0.58	0.73	1.06	0.74	0.98	0.82	3.20
14 days	0.44	0.87	0.7	1.18	1.98	1.03	1.72
21 days	0.96	-	1.98	1.60	2.12	1.65	1.98

(*) All the values are multiplied x 10.

Table A 5. Fresh root weight (percent of control), of plants of the cultivar Conrad inoculated with a zoospore suspension (2.7×10^4 /ml of P. m. glycinea. race 4, in the growth chamber experiment.

Age at inoculation	I	II	III	IV	V	Av.
2 days	0.0	27.8	0.0	0.0	0.0	5.6
4 days	13.3	13.3	0.0	0.0	0.0	5.3
6 days	4.1	0.0	9.5	0.0	0.0	2.7
8 days	16.1	18.1	20.8	10.7	17.5	16.6
10 days	54.5	27.3	9.8	46.2	103.0	48.2
12 days	18.1	22.4	33.1	23.1	30.6	25.5
14 days	9.3	18.4	14.8	25.0	41.9	21.9
21 days	48.5	-	100	80.8	107.1	84.2

Table A 6. Foliar dry weight (g) of inoculated and control (Ck.) plants of the cultivar Conrad in the growth chamber experiment. Inoculation was made with a zoospore suspension (2.7×10^4 /ml) of *P. m. glycinea* race 4.

Plant age at inoculation	I	II	III	IV	V	Av.	Ck.
2 days	0.00	0.05	0.00	0.00	0.00	0.01	0.15
4 days	0.06	0.08	0.00	0.00	0.00	0.03	0.23
6 days	0.02	0.00	0.03	0.00	0.00	0.01	0.58
8 days	0.42	0.37	0.33	0.15	0.19	0.29	0.8
10 days	0.51	0.20	0.61	0.48	0.52	0.46	0.6
12 days	0.45	0.43	0.78	0.54	0.49	0.54	0.97
14 days	0.42	0.45	0.50	0.81	0.52	0.54	1.35
21 days	0.69	0.20	1.09	1.18	0.61	0.75	1.39

Table A 7. Foliar dry weight (% of Ck) of plants of the cultivar Conrad inoculated with a zoospore suspension (2.7×10^4 /ml) of *P. m. glycinea*. race 4. Growth chamber experiment

Plant age at inoculation	I	II	III	IV	V	Av. (%)	Ck. (g)
2 days	0.0	33.3	0.0	0.0	0.0	6.7	0.15
4 days	24.3	34.8	0.0	0.0	0.0	11.8	0.23
6 days	3.1	0.0	5.5	0.0	0.0	1.7	0.58
8 days	52.5	46.2	41.2	18.7	23.7	36.5	0.8
10 days	85.0	33.3	101.6	80.0	86.9	77.3	0.6
12 days	46.4	44.3	80.4	55.7	50.5	55.5	0.97
14 days	31.1	33.3	37.0	60.0	38.5	40.0	1.35
21 days	49.6	14.4	78.4	84.9	43.9	54.2	1.39

Table A 8. Disease ratings of inoculated (I, II, III, IV) and control (Ck) plants of the cultivar Sloan 8 days after inoculation with a zoospore suspension (7.1×10^3 /ml) of P. m. glycinea. race 4. Each number is the average of five plants.

Plant age at inoculation	I	II	III	IV	Av.	Ck.
5 days	5	6	6	5	5.5	1
8 days	3	3.6	2	2.6	2.8	1
15 days	5	4	1.6	3	3.4	1
22 days	4	2	1.8	1.2	2.3	1
29 days	2	1	1.8	3.2	2.0	1
36 days	1	1	1	1.2	1.0	1
43 days	1	2.6	4.2	3.4	2.8	1
50 days	1.8	2.6	5	5	3.6	1

Table A 9. Disease rating of inoculated (I, II, III, IV) and control (Ck) plants of the cultivar Sloan, 40 days after inoculation with a zoospore suspension (7.1×10^3 /ml) of *P. m. glycinea* race 4.

Plant age at inoculation	I	II	III	IV	Av.	Ck.
5 days	5.0	6.0	6.0	5.4	6	1
8 days	3.0	2.0	2.0	4.0	3	1
15 days	5.4	4.8	2.0	4.0	4	1
22 days	4.0	3.6	3.6	3.6	4	1
29 days	3.6	3.6	3.0	3.4	3	1
36 days	3.0	4.0	4.0	3.0	4	1
43 days	4.0	4.0	5.4	5.4	5	1
50 days	4.0	5.4	5.0	5.0	5	1

Table A 10. Number of plants of the cultivar Sloan remaining alive 40 days after inoculation with a zoospore suspension (7.1×10^3 /ml) of *P. m. glycinea* race 4 in the green house experiment.

Plant age at inoculation	I	II	III	IV	%	Ck.
5 days	1	0	0	1	10	5
8 days	3	4	4	3	70	5
15 days	1	2	4	2	45	5
22 days	2	4	4	4	70	5
29 days	4	5	4	3	80	5
36 days	5	5	5	5	100	5
43 days	5	3	2	2	60	5
50 days	5	1	3	4	65	5

Table A 11. Foliar dry weight of inoculated (I, II) and control (Ck.) plants of the cultivar Sloan, 40 days after inoculation with zoospore suspension (7.1×10^5 /ml) of *P. m. glycinea*, race 4 in the green house experiment.

Plant age at inoculation	I	II	Av.	Ck.
5 days	0.0	0.0	0.0	0.8
8 days	0.3	0.5	0.4	0.3
15 days	0.7	0.4	0.5	0.8
22 days	1.0	1.0	0.9	0.7
29 days	1.0	0.6	0.8	2.7
36 days	4.0	3.9	3.9	3.9
43 days	6.0	5.2	5.5	8.4
50 days	8.4	5.3	6.8	17.0

Table A 12. Foliar dry weigh (g) of inoculated and control (Ck.) plants of the cultivar Dassel, 40 days after inoculation with a zoospore suspension (7.1×10^5 /ml) of *P. m. glycinea* race 4 in the green house experiment.

Plant age at inoculation	I	II	III	IV	Av.	Ck.
5 days	0.6	0.7	0.4	0.6	0.6	0.5
8 days	0.6	1.1	0.6	0.6	0.7	0.8
15 days	1.2	0.7	0.3	0.8	0.8	0.5
22 days	0.8	0.7	0.6	0.9	0.7	1.1
29 days	0.9	1.6	1.1	0.7	1.1	0.9
36 days	2.9	2.1	4.4	1.2	2.6	3.7
43 days	11.9	6.9	7.1	8.2	8.5	9.4
50 days	10.3	9.0	7.5	11.0	9.4	8.6

Table A 13. Analysis of Variance Table of Disease ratings for the cultivar Conrad in the growth chamber experiment.

Function: Anova-2

Data case: 1 to 40

Two-way Analysis of Variance over variable 1 (Blocks) with values from 1 to 5 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	4	0.73	0.181	0.32	0.8589
Treatments	7	133.6	19.089	34.17	0.0000
Error	28	15.64	0.559		
Non-additivity	1	0.65	0.645	1.16	0.2907
Residual	27	15.0	0.555		
Total	39	149.99			

Gran Mean = 3.435 Grand Sum = 137400 Total Count = 40

Coefficient of Variation = 21.76 %

Table A 14. Analysis of Variance Table of percent of living plants of the cultivar Conrad 20 days after inoculation in the growth chamber experiment.

Function: Anova-2

Data case 1 to 40

Two-way Analysis of Variance over variable 1 (Blocks) with values from 1 to 5 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	4	160.0	40.0	0.10	0.98
Treatments	7	57030.0	8147.14	21.28	0.000
Error	28	10720.0	382.857		
Non-additivity	1	96.01	96.013	0.24	
Residual	27	10623.9	393.481		
Total	39	67910.0			

Grand Mean = 56.500 Grand Sum = 2260.0 Total Count = 40

Coefficient of Variation = 34.63 %

Table A 15. Analysis of Variance of Fresh root weight as a percent of the control plants of the cultivar Conrad in the growth chamber experiment.

Function: Anova-2

Data case: 1 to 40

Two-way Analysis of Variance over variable 1 (Blocks) with values from 1 to 5 and variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Block	4	1414.68	353.669	1.30	0.294
Treatments	7	26830.86	3832.98	14.09	0.00
Error	27	7342.52	271.945		
Total	38	35588.06			

Grand Mean = 26.248 Grand Sum = 1049.935 Total Count = 40

Coefficient of Variation = 62.83 %

Table A 16. Analysis of Variance Table of Foliar dry weight as a percent of the control plants of the cultivar Conrad in the growth chamber experiment.

Function: Anova-2

Data case: 1 to 40

Two-way Analysis of Variance over variable 1 (Block) with values from 1 to 5 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	4	942.23	235.582	0.71	0.589
Treatments	7	25263.39	3609.05	10.94	0.000
Error	28	9234.21	329.79		
Non additivity	1	2588.74	2588.74	10.52	0.003
Residual	27	6645.47	246.128		
Total	39	35439.92			

Grand Mean = 35.459 Grand Sum = 1418.34 Total Count = 40

Coefficient of Variation = 51.22 %

Table A 17. Analysis of Variance Table of Disease ratings 8 days after plant inoculation of the cultivar Sloan in the greenhouse experiment.

Function: Anova-2

Data case: 1 to 32

Two-way Analysis of Variance over variable 1 (Block) with values from 1 to 4 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	3	0.09	0.031	0.03	0.9945
Treatments	7	50.72	7.246	5.82	0.0008
Error	21	26.16	1.246		
Non-additivity	1	0.77	0.773	0.61	
Residual	20	25.38	1.269		
Total	31	76.97			

Grand Mean = 2.969 Grand Sum = 95.00 Total Count: 32

Coefficient of Variation = 37.59 %

Table A 18. Analysis of Variance Table of Disease ratings 40 days after plant inoculation of the cultivar Sloan in the greenhouse experiment.

Function: Anova-2

Data case: 1 to 32

Two-way Analysis of Variance over variable 1 (Blocks) with values from 1 to 4 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	3	0.79	0.262	0.49	0.695
Treatments	7	23.36	3.336	6.20	0.000
Error	21	11.30	0.538		
Non-additivity	1	0.18	0.177	0.32	
Residual	20	11.12	0.556		
Total	31	35.44			

Grand Mean = 4.088 Grand Sum = 130.80 Total Count = 32

Coefficient of Variation = 17.94 %

Table A 19. Analysis of Variance Table of Foliar dry weight as a percent of the control plants of the cultivar Sloan in the greenhouse experiment.

Functio: Anova-2

Data case:1 to 16

Two-way Analysis of Variance ove variable 1 (Block) with values from 1 to 2 and over variable 2 (Treatments) with values from 1 to 8.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Prob
Blocks	1	19.69	19.961	0.04	0.844
Treatments	7	37831.87	5404.55	11.46	0.002
Error	7	3302.08	471.726		
Non-additivity	1	1040.70	1040.69	2.76	0.147
Residual	6	2261.38	376.897		
Total	15	41153.64			

Grand Mean = 73.934 Grand Sum = 1182.95 Total Count = 16

Coefficient of Variation: 29.38

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