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ABSTRACT

MECHANISM OF INCREASED ROOT ROT IN VIRUS-INFECTED PEAS

by Marvin K. Beute

The mechanism of increased fungal root rot induced by virus infection in peas was studied. Two mechanisms are possible: 1) The inherent susceptibility of root tissue is increased by virus infection; 2) Increased leakage of nutrients from roots of virus-infected plants increases the inoculum potential of the fungi in the rhizosphere.

Increased Fusarium root rot in peas (Pisum sativum L. cv. Miragreen) infected with bean yellow mosaic virus (BYMV) could not be explained by changes in inherent susceptibility. When roots were subjected to hourly leaching, Fusarium root rot was equally severe in both virus-infected and non-virus-infected plants. Addition of exudates from roots of virus-infected plants to plants inoculated with F. solani f. pisi gave more severe root rot than did root exudates from healthy plants.

Roots of BYMV-infected pea plants growing in water

or sand cultures released more electrolytes, nucleotides, amino acids and carbohydrates than did healthy roots. Increased exudation began about 4 days after virus infection and ended after 12-16 days. Total amino compounds in the exudate increased more than did total carbohydrates or nucleotides. Similar results were obtained with pea infected with common pea mosaic virus (PVM) and pea enation mosaic virus (PEMV). Increased root rot induced by virus infection in peas occurred only during the period of increased exudation from roots of virus-infected peas.

Root exudates from virus-infected plants contained more substances supporting growth of F. solani than occurred in exudates from healthy plants. Also, germination of Helminthosporium victoriae conidia on the surface of natural soil was greater adjacent to roots of pea plants infected with BYMV 8 to 16 days previously than adjacent to healthy plants. This period of increased germination coincided with the period of increased root exudation from roots of virus-infected plants.

Each of 10 identified amino acids was released in greater quantities from roots of BYMV-infected plants than from healthy roots. Alanine exudation was increased the most following virus infection. Threonine was the most

prominent amino acid in root exudates from both virus-infected and healthy Miragreen plants. Phenylalanine was present in root exudates from virus-infected plants, but not from healthy plants. The increase in exudation of amino acids from roots of virus-infected plants could not be explained by changes in total quantities or in specific amino acids within root tissue.

Roots of BYMV-infected Miragreen peas immersed in a buffer solution containing $^{32}\text{PO}_4$ accumulated more ^{32}P than did healthy roots. No selective decrease in accumulation of radioactivity was noted with a 2 hour pretreatment of roots with 2,4-dinitrophenol, an uncoupler of oxidation from phosphorylation. Increased uptake by roots of virus-infected plants suggests an increase in plasma membrane permeability, which may also account for the increased leakage of substances from roots of virus-infected plants.

Fusarium root rot was increased by adding the prominent amino acids found in virus-infected root exudates to fungus-inoculated plants, but neither glucose nor selected organic acids increased disease.

The following explanation is proposed for the mechanism of increased root rot in virus-infected pea: Virus infection causes changes in membrane permeability

of root cells, resulting in increased exudation of many kinds of compounds, including nutrients utilizable by the fungus. This increased supply of nutrients increases the inoculum potential of the pathogen in the rhizosphere, which in turn, results in increased root rot.

Two experimental pea varieties, each possessing moderate resistance, one to *Aphanomyces* root rot, and the other to both *Aphanomyces* and *Fusarium* root rots, released more carbohydrates but less amino acids from their roots than did the susceptible variety Miragreen. Root leachings from varieties resistant to either fungus failed to increase root rot when added to Miragreen plants inoculated with the same fungus. However, when root leachings from the *Aphano-**myces* resistant variety were added to *Fusarium*-inoculated Miragreen plants, severity of root rot was increased to the level of disease in plants receiving root leachings from Miragreen plants. These results suggest that the level of resistance observed in the experimental pea varieties may be, in part, a reflection of the influence of root exudates on inoculum potential in the rhizosphere.

Infection with *F. solani* f. *pisi* caused an early increase in infectivity of BYMV or PMV in pea leaf sap. A 2-3 fold increase in local lesions was noted using BYMV +

F. solani-infected leaf sap beginning 5 days after inoculation. Beyond 12 days from initial inoculation, however, no differences in virus titer were found. Infection with F. solani resulted in a 2-3 fold increase in PMV infectivity between the third and fourth day after inoculation. Thereafter, leaf sap from virus + fungus-infected plants were equally infective.

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IN VIRUS-INFECTED PEAS

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INTRODUCTION

Previous studies at Michigan State University indicated that viruses may play a role in development of the fungus root rots of pea (Pisum sativum L.) (12, 13). Over a wide range of greenhouse environmental conditions, 3 pea varieties were more susceptible to Aphanomyces or Fusarium root rots when plants were inoculated previously with either common pea mosaic virus, bean yellow mosaic virus, alfalfa mosaic virus or pea enation mosaic virus. Similar results were obtained with different strains of 2 of the viruses, plants of different ages, and when different time intervals between virus and fungus inoculation were used.

My research problem was to study the mechanism of increased root rot associated with virus infection. Two mechanisms are possible: 1) The inherent susceptibility of root tissue is increased by virus infection; 2) Increased leakage of nutrients from roots of virus-infected plants increases the inoculum potential of the fungi. Farley (12) found that when residual fungal inoculum was removed from

surface of the roots at the time of virus inoculation 3-5 days after fungal inoculation, no increase in root rot occurred above that in fungus-only-infected plants treated in the same manner. This suggested that susceptibility of virus-infected root tissue to the root rot fungi was not changed.

Therefore, most of the research reported herein was directed toward evaluating the role of root exudates as the primary factor in the increased root rot. Root exudates from healthy and virus-infected plants were analyzed to determine any changes in quality or quantity of nucleotides, carbohydrates, amino acids or organic acids brought about by virus infection. The effect of the root exudates on growth of Fusarium solani f. pisi (F. R. Jones) Snyder and Hansen and on disease development was also studied.

LITERATURE REVIEW

INTRODUCTION: Exudation of soluble organic substances is a phenomenon common to root systems of all higher plants. The importance of root exudates in influencing soil microorganisms, both pathogens and saprophytes, has been demonstrated for many plants. A major source of exudates from uninjured seedlings appears to be intact cells located in the region of highest enzymatic activity in the root, i.e., in the vicinity of root tips (47, 49). Other major sites of exudation appear where breaks occur in the epidermis (58). The picture emerging from knowledge of the types of substances found in root exudates is that most substances involved in the cellular metabolism of higher plants are released by exudation.

Results with pea and oat seedlings indicate that exudates are a more important source of organic materials in the rhizosphere than cell debris. However, as the plant develops, exudation declines and decomposition of moribund root hairs, and epidermal and cortical cells contribute a substantial portion of the nutrients to the rhizosphere

(50). Amino acids form the most studied group of compounds in root exudates. The zone of rapid cell elongation immediately behind the growing tip is the most active amino acid-exuding area (47, 58). The following amino acids are known to be liberated by pea roots: glutamic and aspartic acids, proline, leucine, alanine, cysteine, glycine, lysine, phenylalanine, asparagine, serine, glutamine, homoserine, tryptophane, methionine/valine, γ -amino butyric acid, and threonine (25, 50).

The sugars glucose and fructose were found in pea root exudate during the first 10 days of growth (50). However, when sand and root washings of plants were examined after 21 days of growth, the sugars were no longer detected. Although organic acids are found inside the root at significant levels, there are few reports of these materials occurring in root exudates (53). The presence of vitamins in root exudates has long been suspected (34), but the number and level of vitamins reported have been very low. Meshkov (41) reported biotin and thiamin in the exudate from pea and corn roots. The purines adenine and guanine (37), the nucleotides uridine and cytidine (15) as well as 3-4-dioxyflavonone have been found in the root exudates from pea (37). Krasil'nikov (28) found that exudates from

peas contained the enzymes invertase, amylase and protease.

Root exudation is affected by many factors.

Katznelson et al. (25, 26) showed that successive drying and remoistening of peas, soybean, wheat, barley and tomatoes growing in sand and soil-sand mixtures greatly increased the release of amino acids from roots. Exudation mixtures greatly increased the release of amino acids from roots. Exudation was also greatest under high temperatures and light intensity conditions (51). Addition of fertilizer increases the rhizosphere populations of most plants, indicating a greater leakage of nutrients from roots of well fertilized plants (52). Agnihotri (1) reported increases in the exudation of glutamine, γ -amino butyric acid and other amino acids from roots following the foliar application of urea sprays. Urea sprays also caused increased glucose and fructose exudation, but organic acid exudation decreased.

Rovira postulated that microorganisms can affect exudate patterns by altering the permeability of root cells, by modifying the metabolism of roots, and by modifying some of the materials released from roots (53). The amino acid patterns in exudates from tomato and clover roots were shown to be altered by microorganisms in the rhizosphere (51). Exudation is also influenced by disease, and

exudation of nutrients from diseased tissues may in some cases make infection autocatalytic or progressive (57).

EFFECTS OF VIRUS INFECTION ON ROOT EXUDATION:

Lesions resulting from infection by either Fusarium solani f. phaseoli or tobacco necrosis virus on hypocotyls of bean or cowpea exuded high levels of amino acids and reducing sugars (57). Such lesions are necrotic and would be expected to release cellular constituents. However, systemic virus infection without local lesions may also result in changes in root exudation. In Dolichos lablab infected with dolichos enation mosaic virus, changes in physiology of virus infected tissue were accompanied by a change in rhizosphere microfloras (31, 54). Virus infection altered not only the rate of increase of organisms in the rhizosphere, but also the time of manifestation of maximum rhizosphere effect. Between 1 and 5 days after inoculation, fungal numbers tended to increase. Bacteria and actinomycetes increased between 5 and 15 days after virus inoculation. Twenty days after virus infection, the total microbial numbers were considerably reduced as compared with those from the healthy plant. In an investigation of the physiological changes which precede tobacco etch virus-induced wilt of Tabasco pepper, Gabriel (16) showed

that a marked release of electrolytes from roots occurred 24-36 hr before the onset of wilt and 12 hr before respiratory changes could be detected. No such release of electrolytes occurred with noninoculated plants nor with wilt resistant, but virus susceptible plants infected with tobacco etch virus. Stained sections of roots of infected Tabasco seedlings showed no phloem or cambium necrosis at the time of electrolyte release.

VIRUS-FUNGUS INTERACTIONS: Associative effects of virus infection on fungus disease development can be outlined as follows: 1) Fungal infection is increased, decreased or not effected in virus-infected plants. 2) Virus infection is increased, decreased or not effected in fungus-infected plants.

Fungus infection is increased: A number of reports indicate that fungal infections of foliage and roots of plants are increased when the plants are also infected with a virus. Early blight (Alternaria solani) symptoms on leaves of several potato varieties in the field were usually more severe on mosaic-infected plants than on mosaic-free plants (23). Field observations indicated that the late blight fungus (Phytophthora infestans) was present in about twice as many tubers of the potato variety Ulster Supreme

infected with the leaf-roll virus than in tubers of virus-free plants (48). Rice plants infected with hoja blanca virus were more susceptible to Cochliobolus miyabeanus than non-virus-infected rice plants (30).

Post-emergence damping-off of cucumber caused by Rhizoctonia sp. increased as a result of cucumber mosaic virus infection (3). Similarly, sudden wilt of cucumber in Israel was reported to be a result of synergism between Pythium ultimum and cucumber mosaic virus (46). In 1964, Farley and Lockwood found that 3 pea varieties were more susceptible to 2 common fungus root rot diseases when plants were inoculated previously with any one of 4 different viruses (13). Shortly thereafter, Watson et al. (64) described the importance of the virus-fungus interrelationship in reproducing the clover decline-root rot syndrome as it occurs in Idaho. They also reported a severe root rot epidemic on pea associated with widespread virus infection in the field. Fusarium and Rhizoctonia root rots of wheat in New Zealand and Canada occurred predominantly in plants infected with barley yellow dwarf virus (59). Williams and Alexander noted that a higher incidence of root and stalk rot occurred in corn infected with maize dwarf mosaic virus (65). Virus-infected, greenhouse-grown Ohio W49 yellow

dent corn, Golden Bantam sweet corn and Lucas wheat were more susceptible to root rots, stalk rots, and seedling blights (45) than were plants free of virus.

Fungus infection is decreased: In contrast to the positive interactions discussed above, Muller and Monro (44) observed that the growth Phytophthora infestans was retarded on leaves of potato plants previously inoculated with virus X or virus Y. Similarly, reduced susceptibility of cucumber to powdery mildew (Erysiphe cichoracearum) or scab (Cladosporium cucumerinum) infection (24) occurred when cucumber mosaic virus was present in the plant. Tobacco mosaic virus infection reduced susceptibility of Pinto bean (Phaseolus vulgaris) to bean rust (Uromyces phaseoli typica) (67), and leaf-roll virus reduced susceptibility of grape to powdery mildew (Uncinula necator) (19). Infection of pigeon pea (Cajanus cajan) with sterility virus afforded protection against Fusarium-wilting (9), and infection of red clover (Trifolium pratense) with bean yellow mosaic virus retarded subsequent infection by powdery mildew (Erysiphe polygoni) (27).

Fungus infection is not affected: Systemic infection by tobacco mosaic virus did not alter the susceptibility of Nicotiana tabacum var. Turkish leaf tissue to Thielaviopsis basicola (21). However, a localized, nonspecific resistance

to T. basicola was induced in Samsun N N tobacco half-leaves opposite half-leaves inoculated with TMV.

Losses from Fusarium oxysporum, F. roseum (F. avenaceum), Rhizoctonia solani and Sclerotium bataticola were neither increased or decreased on red clover infected with bean yellow mosaic virus (39). Fusarium sp. and Rhizoctonia sp. were isolated with equal frequency from white clover plants infected with bean yellow mosaic virus, red clover virus, pea mosaic virus, and from virus-free plants (20). However, the actual susceptibility of the plants to these pathogens was not determined by these investigators.

Virus infection is increased: The concentration of 11 viruses in sap from bean leaves infected with Uromyces phaseoli appeared to be increased compared with that in non-rusted tissue (18, 68). Based on local lesion assays, addition of rusted bean tissue (0.003% - 0.01%) to TMV sap increased local lesions by 77%. When 3-day old rust mycelia in Pinto bean leaves were killed by heat and the treated tissue inoculated with TMV up to 4 days after therapy, a similar increase in virus concentration occurred. Sunflower (Helianthus annuus) leaves infected with rust (Puccinia helianthi) had higher concentrations of 6 viruses than did

non-rusted leaves. Since Erysiphe polygoni or Colletotrichum lindemuthianum, which enter cells of bean by direct penetration, did not favor virus infection, increased susceptibility of rusted tissue to virus infection did not appear to be due to the punctures made by rust haustoria.

Virus infection is decreased: Resistance against TMV was acquired by tobacco plants (Nicotiana tabacum) previously infected with Thielaviopsis basicola (5). Virus lesions were smaller in size and fewer in number when the lower leaves of Samsun tobacco were first inoculated with T. basicola, then followed 7 days later by a challenge inoculation of the upper leaves with TMV.

Tobacco plants (N. tabacum) with Thielaviopsis basicola lesions on stem or leaf tissue developed systemic resistance to tobacco necrosis virus (21). However, root infection by the fungus did not alter susceptibility of aerial plant parts to virus infection.

Based on the literature reviewed above, one can conclude that diseases caused by obligate parasites are often decreased by virus infection of the host. Examples of this generalization include rusts and powdery mildews, which are considered to be the most specialized or advanced pathogens (40). On the other hand, fungal diseases caused by

primitive parasites are commonly increased by virus infection of the host. These include damping-off and root rot diseases. In some cases, failure to detect any change in a fungal disease when the same plant is also infected with a virus was due to use of inadequate methods for assessing such changes.

MATERIALS AND METHODS

Source and maintenance of fungus and virus cultures:

Fusarium solani f. pisi (F. R. Jones) Snyder and Hansen, isolate 19 was obtained by isolation from a Michigan pea field. Aphanomyces euteiches Drechs. isolate 4 was obtained from W. T. Schroeder, Geneva, N. Y., and F. oxysporum f. pisi (Linford) Snyder and Hansen, race 2, isolate 90 was obtained from D. J. Hagedorn, Madison, Wisconsin. Bean yellow mosaic virus (BYMV) was supplied by W. T. Schroeder, common pea mosaic virus (PMV) was supplied by D. J. Hagedorn and pea enation mosaic virus (PEMV) was supplied by J. Bath, Michigan State University.

F. solani f. pisi, F. oxysporum f. pisi race 2, and A. euteiches were maintained on potato-dextrose agar (PDA) slants at 24°C. All fungi were pathogenic on pea. Common pea mosaic virus, bean yellow mosaic virus and pea enation mosaic virus were maintained in a greenhouse (70-90°F) by periodic sap inoculation on pea variety Miragreen. Viruses in infected, finely-chopped pea leaves were also stored over CaCl₂ in a freezer.

Growing plants: A commercial pea (Pisum sativum L., cv. Miragreen) was used as a host plant unless specified otherwise. Pea seeds were surface sterilized by soaking in 0.5% sodium hypochlorite (10% Clorox) for 15 minutes, followed by rinsing with distilled water. Seeds were planted in silica sand or in an autoclaved mixture of equal parts sand and ground peat. Usually, 10 seedlings were grown in moist silica sand in 4 in. plastic pots (1,400 g sand/pot). However, seedlings were also grown in 8 in. diameter glazed crocks, each with a hole on the side near the bottom, to allow drainage of excess fluid from the sand.

For collection of root exudates, plants were first grown in silica sand in 4 in. plastic pots. When seedlings were 1-2 in. high, plants were removed from the sand by flooding the pots with water and gently agitating the sand until plants were floated free. Seedlings were placed in 4 in. plastic pots and a slurry consisting of acid washed silica sand and water was used to fill the pots.

In experiments designed to remove external fungal inoculum from plants, seedlings were first grown in moist silica sand in 4 in. plastic pots or in rows in metal pans. Healthy plants and plants inoculated with a fungus 3 days previously were carefully removed from the sand, and roots

were rinsed several times in running tap water before transplanting to soil infested with F. solani or to non-infested soil.

Inoculation procedures: Fungus inoculum was prepared in the following way: F. solani was grown on PDA in petri dishes at 24°C for 14-21 days. Conidia were removed from the agar surface by adding distilled water, followed by gentle agitation. The number of conidia was adjusted to a standard concentration. Inoculum of A. euteiches was prepared from mycelia grown at 24°C in 250 ml Erlenmeyer flasks containing 50 ml of maltose-peptone broth (33). Production of zoospores was induced by allowing 4-6 day old mycelial mats to stand in tap water for 2 hr, followed by aeration in distilled water for 12 hr (33). Zoospores were adjusted to a standard concentration. F. oxysporum was grown on PDA in petri dishes at 24°C. After 14-21 days both mycelia and conidia were scraped from the agar surface and ground with a small volume of distilled water in a Waring Blendor for 1 minute.

Pea plants were inoculated with F. solani conidia or A. euteiches zoospores by pipetting 10 ml of the inoculum suspension uniformly on the surface of the sand or soil in the pot or row in which the peas were growing. The surface

of the sand or soil was immediately watered to saturation with distilled water. Inoculation with F. oxysporum was accomplished in one of two ways: 1) Pea seedlings were removed from the soil, their root systems dipped into the inoculum suspension and the seedlings replanted into autoclaved soil. 2) Twenty ml of inoculum suspension was added to each pot by applying 4 ml portions in each of 5 positions with a hypodermic syringe inserted to a depth of 1 in. below the soil surface.

Virus inoculum was prepared by growing virus-infected Miragreen pea plants in the greenhouse. Leaves of plants inoculated 10-20 days previously were ground in a mortar together with a small amount of 0.1 M phosphate buffer and 400 mesh carborundum powder. Two expanded leaves on each plant were dusted with carborundum and rubbed with a polyurethane swab containing the infected sap. Control leaves were dusted with carborundum and rubbed with a polyurethane swab containing 0.1 M phosphate buffer.

Evaluation of disease: Disease was evaluated 2-3 weeks after fungus inoculation by estimating the severity of disease in foliage, epicotyls and roots (35). Tops of plants with only the lower one or two leaves wilted were rated 1, completely wilted tops were rated 3 and intermediate

stages were rated 2. Watersoaked A. euteiches-infected collars (area immediately above and below seed attachment) were rated 1 and completely collapsed collars 2. Slight, moderate and severe infection of collars by Fusarium was rated 1, 2 and 3, respectively. Slight, moderate and severe root decay was rated 1, 2 and 3, respectively, for all diseases. The disease index was the sum of ratings for foliage, collars and epicotyls. Thus, disease severity was expressed on a scale of increasing severity from 0-9 for Fusarium root rot and 0-8 for Aphanomyces root rot. Usually, the ratings were made for the group of plants in each pot and not for individual plants.

Collection of root exudates: When seedlings grown in silica sand were 3-4 in. tall, half of the plants were inoculated with a virus, followed by immediate removal of all plants from the silica sand. The plants were treated in either of the following ways: 1) Four-six healthy and virus-infected plants with cotyledons excised were maintained in 100 ml distilled water from the time of virus inoculation through a 12-16 day period. The cultures were grown for 16 hr at 72°C under 520 foot candles of light, followed by an 8 hr dark period at 56°C. The water was changed daily and conductivity of the discarded culture solution was measured.

2) Seedlings were transplanted into acid washed silica sand in 4 in. plastic pots. At 1 hr intervals, 10 ml of 10^{-1} diluted Hoagland's solution (33) was applied to the surface of the sand in which 6-15 seedlings were growing. The leachate drained from holes in the bottom of the container, and was collected in a sufficient volume of ethanol to insure a final concentration of not less than 50% ethanol after a 24 hr collection period (Figure 1). Ethanol was used to prevent utilization of exudates by contaminating organisms.

All solutions containing root exudates were passed through 0.45μ Millipore filters and evaporated to dryness at 60°C in a flash evaporator. The ethanol soluble fraction of the residue was removed and evaporated to dryness. The final residue was resuspended in 10 ml of glass distilled water. Solutions were frozen for storage.

Chemical analyses of exudates: The conductivity of water culture solutions was determined with a conductivity bridge. Results were expressed in mhos, the reciprocal of the electrical resistance of the fluid in ohms. Nucleic acid content of root exudates was determined by ultraviolet absorption (64). Portions of the concentrated solutions containing root exudates were placed in a 3 ml silica

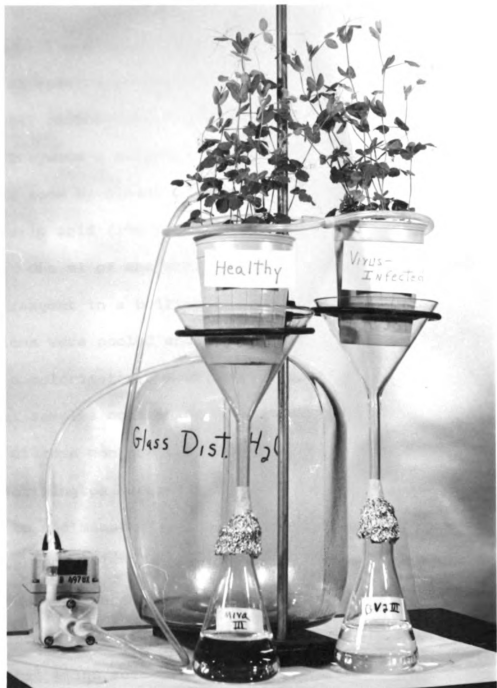


Figure 1. Apparatus used for collecting root exudates of pea growing in silica sand. Ten ml of 10^{-1} diluted Hoagland's solution or distilled water was pumped on the surface of the sand hourly. Leachate drained from holes in the bottom of the container, and was collected in a sufficient volume of 95% ethanol to insure a final concentration of not less than 50% ethanol after a 24 hr collection period.

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absorption cell and absorption at 260 m μ was recorded using a Beckman DB spectrophotometer.

Total carbohydrates were determined using a modification of Dreywood's anthrone reagent (43). The anthrone reagent was made by dissolving 0.4 g anthrone in 200 ml of 9.3 M sulfuric acid (190 ml concentrated sulfuric acid + 10 ml water). One ml of the solution to be assayed was mixed with 2 ml reagent in a boiling water bath for 3 minutes. The solutions were cooled and the optical density read at 620 m μ in a colorimeter. A standard curve was made by measuring 1 ml samples containing 20, 30, 60, 80 and 100 μ g of glucose. Glucose content was determined with the Glucostat reagent (Worthington Biochemical Corporation) prepared according to the manufacturer's directions. Optical density was read at 400 m μ in a colorimeter and compared with a standard curve prepared from samples containing 20, 40 and 80 μ g glucose.

Total amino acids were determined by the method of Moore and Stein (42). A standard curve was made by measuring glycine at concentrations of 4, 8 and 16 μ g/ml water. Protein was determined with the Folin phenol reagent (36). This reagent is prepared in several steps. Reagent C consists of a 50: 1 (v:v) mixture of stock solution 1) 2% Na₂CO₃

in 0.1 N NaOH and stock solution 2) 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium tartrate. This mixture must be made fresh daily from stock solutions 1 and 2. One ml of sample solution was mixed with 1 ml reagent C and allowed to stand for 10 minutes. Then, 0.2 ml Folin-Ciocalteu phenol reagent diluted to 1 N with water was added rapidly to the sample solution and allowed to stand for an additional 30 minutes before determining optical density at 500 m μ in a colorimeter. A standard curve was made by measuring 1 ml samples containing 25, 50 and 100 μg of bovine serum albumin.

Concentrated solutions containing root exudates or root tissue extracts were desalted for paper chromatographic separation of amino acids, organic acids and sugars (60). Three cm³ of cation exchange resin (Bio Rad AG 50W-X8, H⁺ form) was prepared by passing 20 ml of 33% HCl through resin in a column of 1 cm inside diam. Three cm³ of anion exchange resin (Bio Rad AG 1-X8, formate form) was prepared by passing 20 ml of 1 N NaOH through resin in a column of 1 cm inside diam. A sufficient volume of distilled water was then passed through both columns to remove all acid or base from the resin beds. The sample solutions were first passed through the cation exchange resin. The resin was then rinsed with 10 ml glass distilled water. The effluent,

including the rinse, was immediately passed through the anion exchange column. This resin was also rinsed with 10 ml glass distilled water. The effluent from the latter column was designated the neutral fraction. The cation exchange column was eluted with 10 ml of 1 N NH_4OH . The anion exchange column was eluted with 10 ml 6 N formic acid. The neutral, cationic, and anionic fractions were evaporated to dryness in a flash evaporator at 60°C and resuspended in 2.5-6.0 ml 10% n-propanol for spotting on chromatographic paper. All chromatography work was done at $24-27^\circ\text{C}$.

The amino acids present in the cationic fraction were separated on Whatman No. 1 chromatography paper (18 x 22 in.). The mobile phases for the 2-dimensional descending chromatograms were n-butanol: acetic acid: water (3:1:1 v/v), followed by phenol: water (4:1 w/v). The chromatograms were air dried for 8-12 hr between solvent systems. Amino acids were detected by spraying 0.5% ethanolic ninhydrin on the developed chromatograms, then heating at 75°C for 10 minutes. Standards made from known combinations of pure amino acids were included with each run. The spots were cut from developed chromatograms, submerged in 3 ml 50% ethanol for 30 min., filtered through Whatman No. 1 paper into a colorimeter tube, and read at 570 m μ in a colorimeter.

A standard curve for each amino acid (15 and 50 μ g) was prepared on paper in a similar manner.

Portions of the concentrated exudate solutions containing the sugars (neutral fraction) were applied uniformly to Whatman No. 1 paper (9 x 22 in.). Equal volumes of root exudate from healthy and virus-infected plants were analyzed. N-butanol: acetic acid: water (4:1:5 v/v) was used as the solvent in descending one-dimensional chromatography. Chromatograms were dried 8-12 hr and aniline phthalate spray reagent (Brinkmann Instrument Inc.) was used to detect reducing sugars.

Portions of the concentrated exudate solutions containing the organic acids (anion fraction) were applied uniformly to Whatman No. 1 paper (9 x 22 in.). Equal volumes of root exudates from healthy and virus-infected plants were analysed. N-pentanol: 5 N formic acid (1:1 v/v) was used as the solvent in descending one-dimensional chromatography. Chromatograms were air dried for 8-12 hr and sprayed with 0.04% ethanolic brom-phenol blue.

Bioassays of exudates: Several bioassays were utilized: 1) The rate of bacterial multiplication in water cultures of plant roots was studied. Samples from the water culture solution were diluted in distilled water or 0.05 M

phosphate buffer (pH 7.0) to 10^{-5} - 10^{-6} , and 1 ml of each serial dilution was added to 100 mm diam petri dishes. Fifteen ml of soil extract agar (43°C) was added to the petri dishes, the dishes were swirled and incubated at 24°C . After 3-6 days, bacterial colonies were counted. 2) The effect of diluted root exudates on Fusarium solani f. pisi germ tube growth was measured. Washed conidia were added to 1.5 ml diluted exudate (10^{-6} concentrated solution) in 60 mm diam petri dishes and incubated for 12 hr at 24°C . Germ tubes in root exudates from healthy and virus-infected plants were measured microscopically. 3) Germination of Helminthosporium victoriae conidia on natural soil adjacent to roots of healthy and virus-infected plants was determined. Root systems contained within a cellophane membrane or nylon mesh were placed in 150 mm diam petri dishes. Moist, nonsterile loam soil was packed around the roots, the surface of the soil was smoothed and conidia washed 3 times by centrifugation in glass distilled water were applied to the soil surface. After 10-13 hr the spores were stained with phenolic rose bengal and recovered with a solution of polystyrene (32). The dried plastic film was transferred to a drop of mineral oil on a glass slide, covered with a cover glass, and then observed microscopically.

Phosphate uptake: Roots of healthy and virus-infected Miragreen peas grown in silica sand were immersed in 300 ml phosphate buffer (10^{-3} M) containing $\text{H}_3^{32}\text{PO}_4$, or in phosphate buffer containing 10^{-4} M 2,4-dinitrophenol for 2 hr before exposure to $\text{H}_3^{32}\text{PO}_4$. After 1-2 hr exposure to ^{32}P , the plants were removed from the buffer and placed in non-radioactive phosphate buffer (10^{-3} M) for 5 minutes. This rinse was repeated 2 times to remove all exchangeable $^{32}\text{PO}_4$ from the root surface. Whole roots or root tips (2 cm) were removed from both healthy and virus-infected plants. The tissue was weighed, evaporated to dryness in scintillation bottles, and 50 ml scintillation fluid containing 5 g PPO (2,5-diphenyloxazole) and 0.3 g POPOP (1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene)/liter toluene was added. Radioactivity was determined with a Packard Tri Carb liquid scintillation spectrometer.

Assay of virus infectivity in pea leaf sap: Miragreen seedlings were grown in an autoclaved mixture of equal parts sand and ground peat. When seedlings were 8-9 days old, plants were simultaneously inoculated with F. solani or F. oxysporum and PMV or BYMV. Beginning 3 days after inoculations, leaf sap was periodically assayed for virus infectivity. For each leaf sap assay, a 10 mm diam disc of leaf tissue from young, newly expanded leaves was punched from each of

10 plants growing in a pot. The discs were ground with pestle and mortar in 10 ml 0.05 M phosphate buffer (pH 7.0). One ml of the crude sap was then diluted in 9 ml buffer and used to inoculate the leaves of a local lesion host, Chenopodium amaranticolor, Coste and Reyn. Chenopodium plants were grown under shade for 7 days previous to inoculation to produce optimum lesion development. Sap from Fusarium-infected plants and non-fungus-infected plants was assayed on single opposite half-leaves on 10 plants.

RESULTS

External vs. internal effect of virus infection on root rot disease: Experiments were designed to determine whether the increased severity of root rot induced by virus infection was brought about: 1) by a change in the inherent susceptibility of root tissue; or 2) by increased inoculum potential resulting from altered root exudates. The transplanting experiment used by Farley (12) was repeated. Immediately after virus inoculation of plants inoculated 3 days earlier with F. solani, roots of inoculated and uninoculated plants were washed, and plants were transplanted to soil infested with F. solani or to non-infested soil. Disease ratings were made 17 days after transplanting.

Virus + fungus-infected plants had no more root rot than fungus-only-infected plants (5% level) when both were transplanted into non-infested soil (Table 1). However, virus + fungus-infected peas transplanted to infested soil had more severe root rot than transplants infected with a

fungus only. These data confirm previous results using the fungi F. solani and Aphanomyces euteiches and the virus PMV (12). The results of these tests suggest that virus infection enhances the infectivity of the fungal inoculum at the root surface, and that the increased root rot was not due to increased susceptibility of virus-infected tissues.

In an attempt to circumvent the influence of an altered external environment, fungal inoculum was placed directly into cortical tissues of the stem. When Miragreen peas grown in silica sand were 1-2 in. high, 3 of 6 plants in each pot were inoculated with BYMV. Eight days after virus inoculation, all plants were inoculated by injecting a 1 μ l suspension of F. solani (10^6 spores/ml) directly into the epicotyl. Disease severity was determined 21 days after fungal inoculation. Length and severity of lesions were the same (5% level) for virus-infected and non-virus-infected plants (Table 2). This is further support for the conclusion that the increase in root rot was not due to a change in susceptibility of root tissue.

Influence of root exudates on disease development: If root exudates are involved in virus-induced increased root rot, removal of the exuded substances should correspondingly

Table 1. The effect of removal of Fusarium solani inoculum from pea roots after BYMV inoculation on virus-induced root rot^a

Seedling origin	Seedling transplanted into	Disease indices for plants infected with indicated pathogens ^b	
		Fungus only	Fungus +BYMV
Non-infested soil	Infested soil	3.1 a	6.4 b
Infested soil	Infested soil	3.4 a	6.4 b
Infested soil	Non-infested soil	2.8 a	4.2 a

^aMiragreen seedlings infected with the fungus for 3 days and healthy seedlings were inoculated with BYMV; plants were removed immediately, their roots were washed, then seedlings were transplanted into non-infested soil or soil infested with fungus.

^bDisease indices were based on a scale of increasing severity from 0-9. Values followed by the same letter did not differ (5% level).

Table 2. The effect of injecting inoculum into cortical tissue on development of Fusarium root rot in Miragreen pea^a

	Lesion Length mm	Disease Index ^b
Virus-infected plants	1.33	1.93
Non-virus-infected plants	1.26	1.61

^aA 1 μ l suspension of F. solani conida (10^6 /ml) was injected into epicotyls immediately after virus inoculation.

^bDisease indices were based on a scale of increase-severity from 0-3. Each figure is the mean of 39 plants. Values for each treatment did not differ (5% level).

reduce the severity of root disease. When Miragreen seedlings growing in silica sand were 1-2 in. tall, plants in half of the pots were inoculated with BYMV on the 2 lower leaves. Three days after virus inoculation, a 10 ml suspension of A. euteiches zoospores (10^4 /ml) or a 10 ml suspension of F. solani conidia (10^6 /ml) was pipetted on the sand surface. At hourly intervals, 20-40 ml of deionized water or 10^{-1} diluted Hoagland's solution was added to each pot. The solution saturated the sand and the excess fluid flowed out of the pots through perforations in the bottom. Plants grown in silica sand without leaching served as controls. Fourteen days later, plants were removed and disease severity determined.

Root rot severity in virus-infected and non-virus-infected plants subjected to hourly leaching, and in non-leached plants with Fusarium only, was the same (Figure 2) in 5 tests. However, root rot severity in virus-infected plants not subjected to leaching was increased. These results suggest that root exudates play an important role in the mechanism of increased root rot. If this assumption is valid, addition of root exudates from virus-infected plants to plants inoculated with a root rot fungus should result in increased disease.

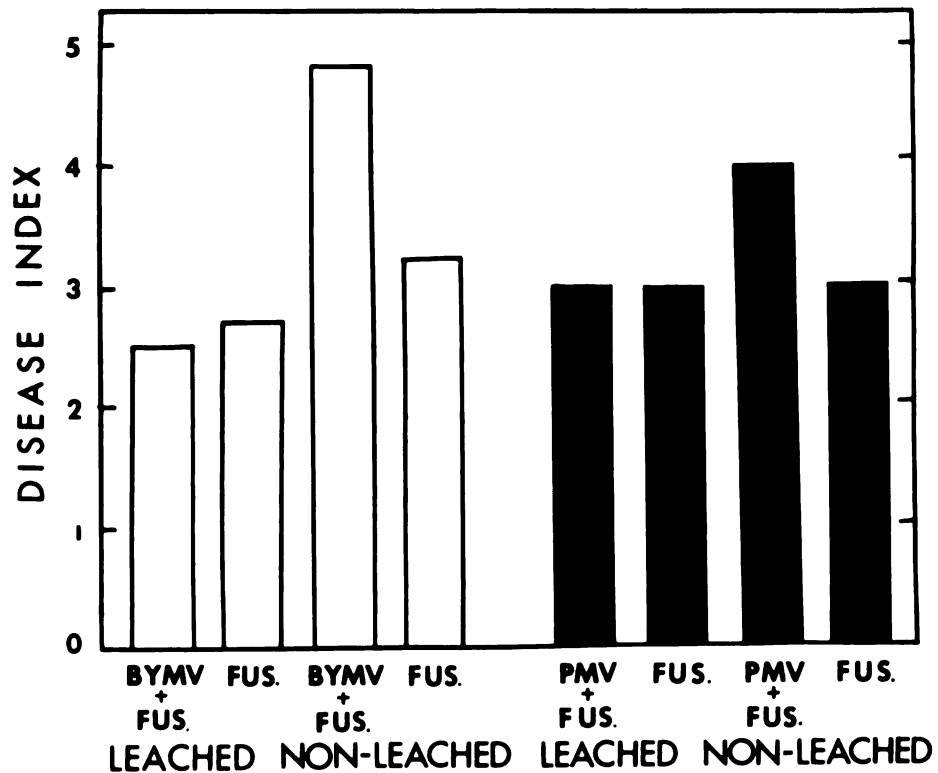


Figure 2. Effect of leaching roots of plants infected with BYMV or PMV on susceptibility to Fusarium root rot. Fourteen days after fungus inoculation, plants were removed and disease severity was determined on a scale of 0-9. The only increased disease occurred in virus + fungus-infected plants not subjected to root leaching. Similar results were obtained with BYMV in 4 additional tests.

Four in. plastic pots containing 10 F. solani-infected Miragreen seedlings growing in silica sand were positioned below the drainage hole in crocks containing healthy or virus-infected Miragreen plants (Figure 3). In an experiment with BYMV, fungus-infected plants received leachings from 10 BYMV-infected or healthy plants. Fifty ml of deionized water was added daily over a 10 day period. In an experiment with PMV, fungus-infected plants received 10 ml of leachate hourly from 10 PVM-infected or healthy plants over a 17 day period. In another experiment with PEMV, fungus-infected plants received 10 ml leachate from 20 PEMV-infected or healthy plants over a 10 day period. Fungus-infected plants receiving leachates from crocks containing only silica sand, and fungus-infected plants receiving no leachates served as controls. Disease severity on fungus-infected plants was determined 17 days after inoculation.

In all experiments, irrespective of the virus used, those plants which received leachates from virus-infected plants had more severe disease (5% level) than plants receiving leachates from healthy plants (Figure 4). Similar results were obtained in 2 additional tests. This evidence

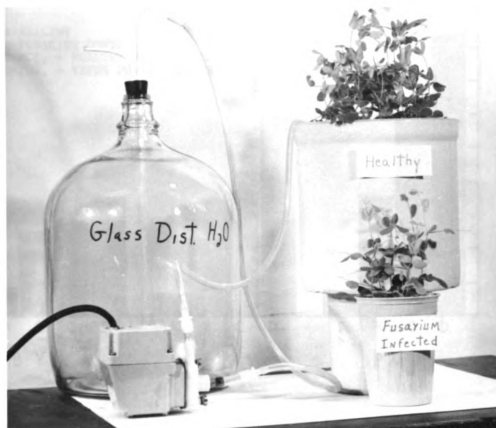


Figure 3. System used to test effects of root exudates from pea roots on *Fusarium* root rot development in peas. At hourly intervals, 20-40 ml of H_2O or 10^{-1} diluted Hoagland's solution was added to the surface of each pot containing healthy or virus-infected plants. Excess fluid flowed out of the pots through perforations in the bottom. Pots containing 10 *F. solani*-inoculated Miragreen seedlings growing in silica sand were positioned below drainage hole in crocks.

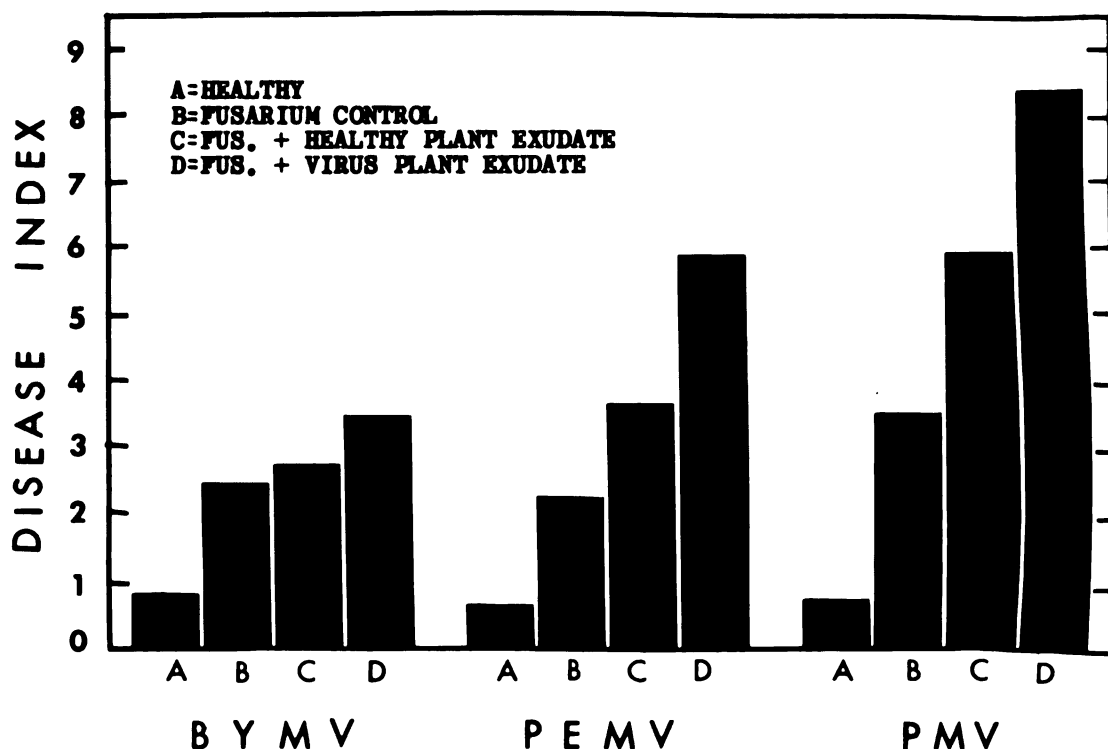


Figure 4. Effect of leachates from pea roots on Fusarium root rot of Miragreen pea. In the experiment with BYMV, plants received leachings from 10 BYMV-infected or healthy plants which were watered with 50 ml of deionized water daily over a 10 day period. In the experiments using PEMV, plants received 10 ml of leachate hourly from 20 PEMV-infected or healthy plants over a 10 day period. In the experiment with PMV, plants received 10 ml leachate hourly from 10 PMV-infected or healthy plants over a 17 day period. Disease severity was determined on a scale of 0-9. In all experiments, plants which received leachates from virus-infected plants had more severe disease (5% level) than plants receiving leachates from healthy plants. Similar results were obtained in 2 additional tests.

is further indication that the mechanism of increased root rot in virus-infected peas is related to root exudates.

Changes in exudation patterns: Water cultures:

Direct evidence of increased release of substances from roots was sought. Root exudates collected from virus-infected and healthy plants grown in water were analyzed at 2-day intervals for electrolytes, carbohydrates, amino acids and nucleotides.

An increased release of electrolytes from roots of BYMV-infected plants, as compared with healthy roots, began 4-6 days after virus infection (Figure 5). Comcomitant with increased electrolyte release from virus-infected roots was an increased nucleotide (Figure 6), carbohydrate (Figure 7) and amino acid (Figure 8) content of the solutions in which virus-infected plants had grown. Similar results were obtained in 2 additional tests. The increased rate of release of these substances continued until 14-16 days after virus inoculation when levels returned to those for healthy plants. The substances detected are assumed to be primarily nutrients not yet assimilated by the microflora in the solutions, and are considered to reflect a differential release of substances by roots of virus-infected and non-virus-infected plants. Of the 4 categories of substances assayed,

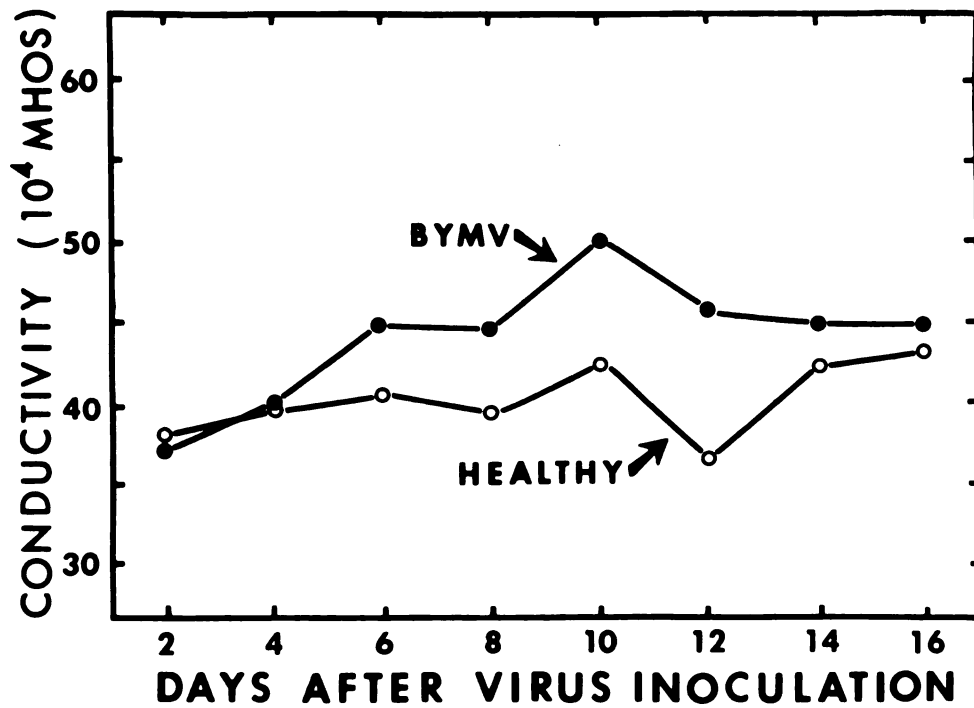


Figure 5. Effect of BYMV-infection on amount of electrolytes exuded by roots of pea plants growing in water cultures. Conductivity of water culture solutions was determined daily. Similar results were obtained in 2 additional tests.

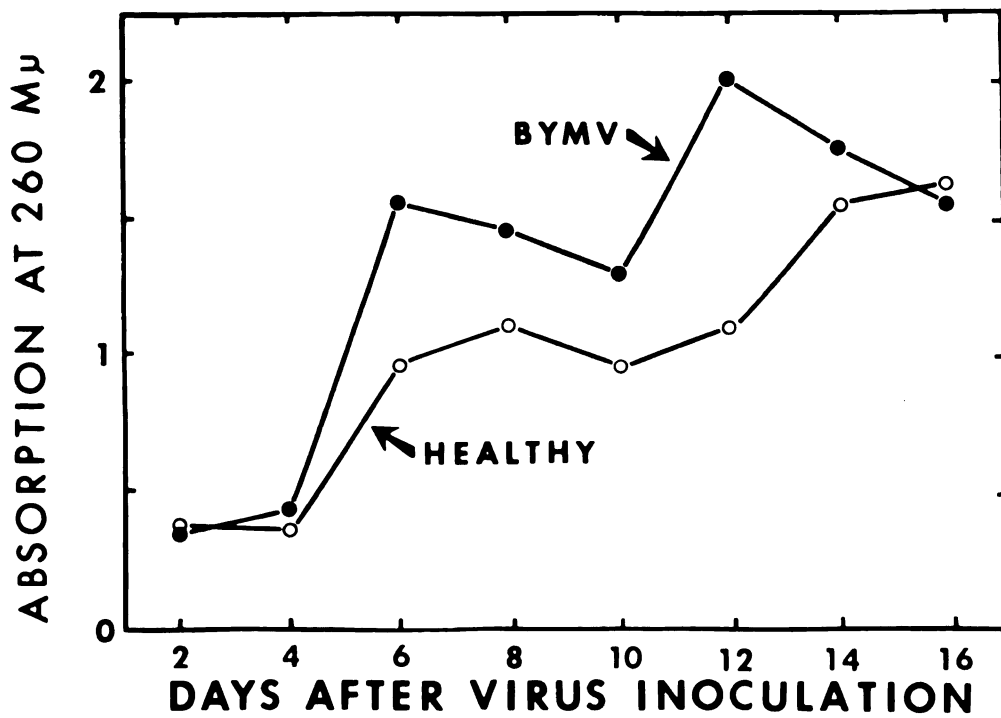


Figure 6. Effect of BYMV-infection on amount of nucleotides exuded by roots of pea plants growing in water cultures. Nucleic acid content was determined by ultraviolet absorption at 260 mμ. Results are from 1 of 3 experiments, all with similar results.

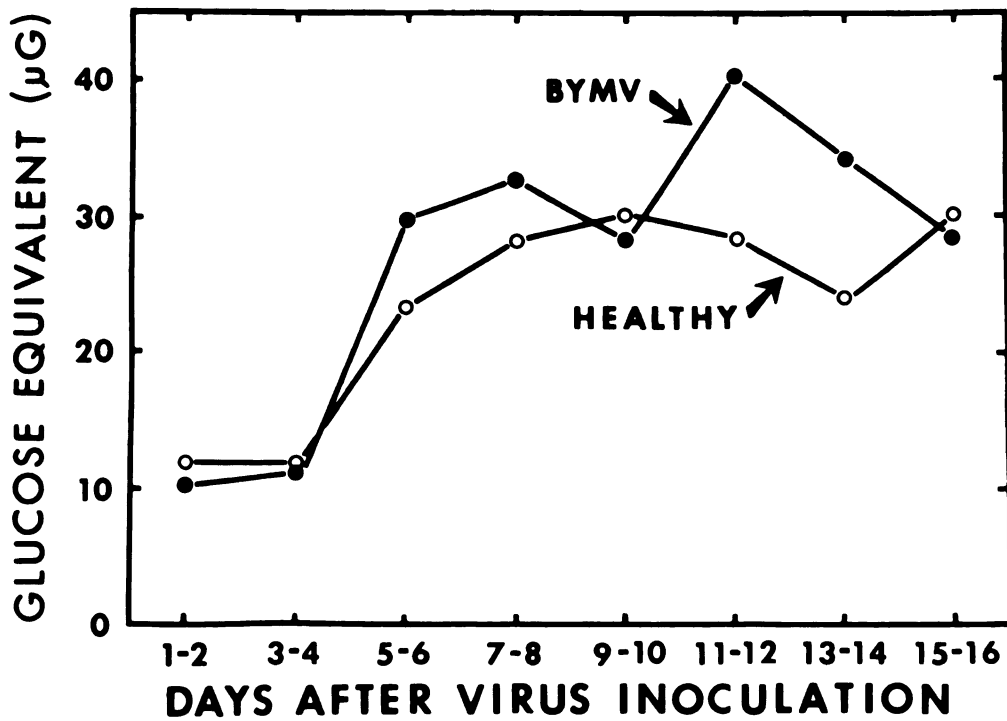


Figure 7. Effect of BYMV-infection on amount of carbohydrates exuded by roots of pea plants growing in water cultures. Carbohydrate content of concentrated exudate solutions was determined using a modified Dreywood's anthrone reagent. Similar results were obtained in 2 additional tests.

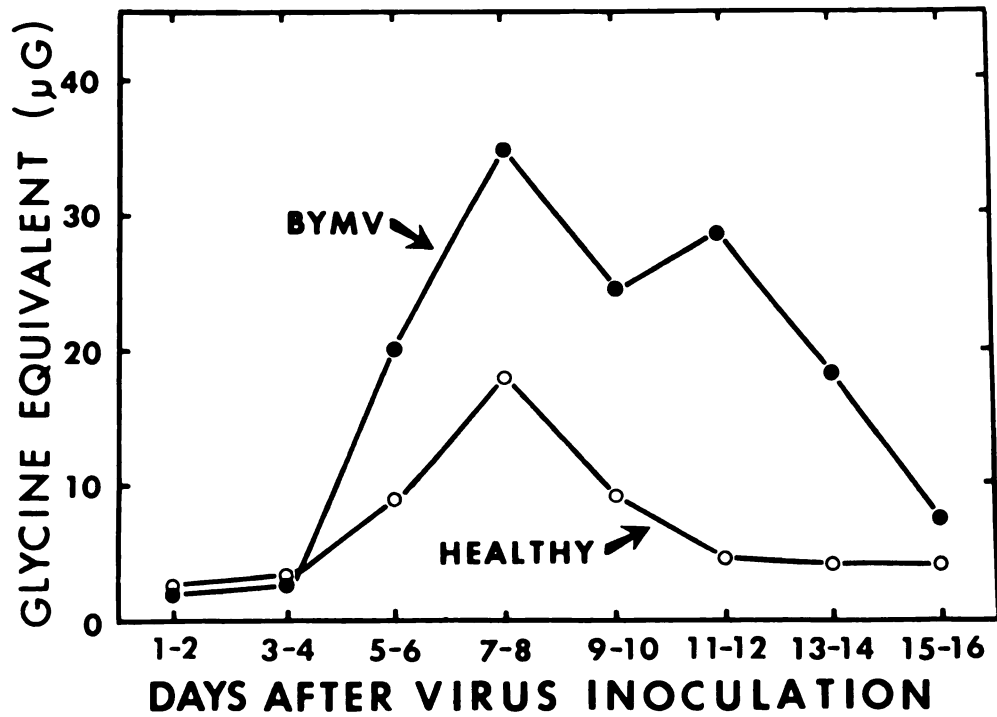


Figure 8. Effect of BYMV-infection on amount of amino acids exuded by roots of pea plants growing in water cultures. Amino acid content of concentrated exudate solutions was determined with ninhydrin reagent. Similar results were obtained in 2 additional tests.

amino acids had the greatest relative increase in exudation (Figure 9). Carbohydrate and amino acid content of water cultures used to grow healthy Miragreen peas showed an approximate release of 25 μ g of carbohydrates and 8 μ g of amino compounds per plant each day. During the period between 4 and 16 days after BYMV infection, a 100-150% increase in amino acids exuded from roots of virus-infected plants can be compared with a 20-40% increase in release of carbohydrates and 30-40% for nucleotides (Figure 9).

Similar increases in exudation of electrolytes, carbohydrates and amino acids were observed with peas infected with PMV and PEMV. The increased release for PEMV began 6-8 days after inoculation and for PMV, 6-10 days after inoculation. The increased exudation had not declined 16 days after virus inoculation with either PEMV or PMV.

Sand cultures: The nucleotide, amino acid, carbohydrate and protein content of root exudates collected from plants grown in acid washed silica sand was determined.

An increased release of amino compounds from roots of BYMV-infected plants began 4-6 days after virus inoculation (Figure 10). The rate of exudation decreased to the level of healthy controls 13-14 days after virus inoculation. Similarly, increased exudation of carbohydrates (Figure 11)

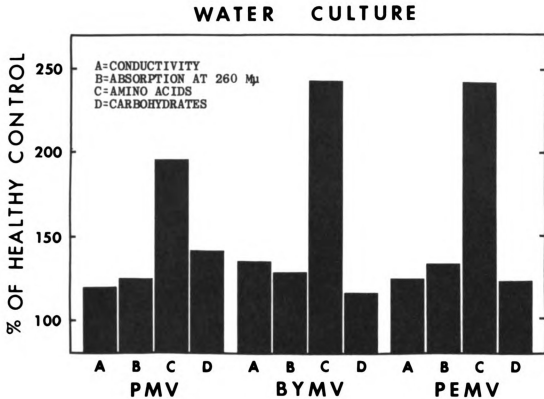


Figure 9. Increased exudation of electrolytes, nucleotides, carbohydrates and amino compounds by virus-infected Miragreen pea plants in water culture relative to the exudation by non-infected plants taken as 100%. Values are the average daily release of each for the period from virus inoculation through 16 days later. Similar results were obtained for BYMV in 4 additional tests.

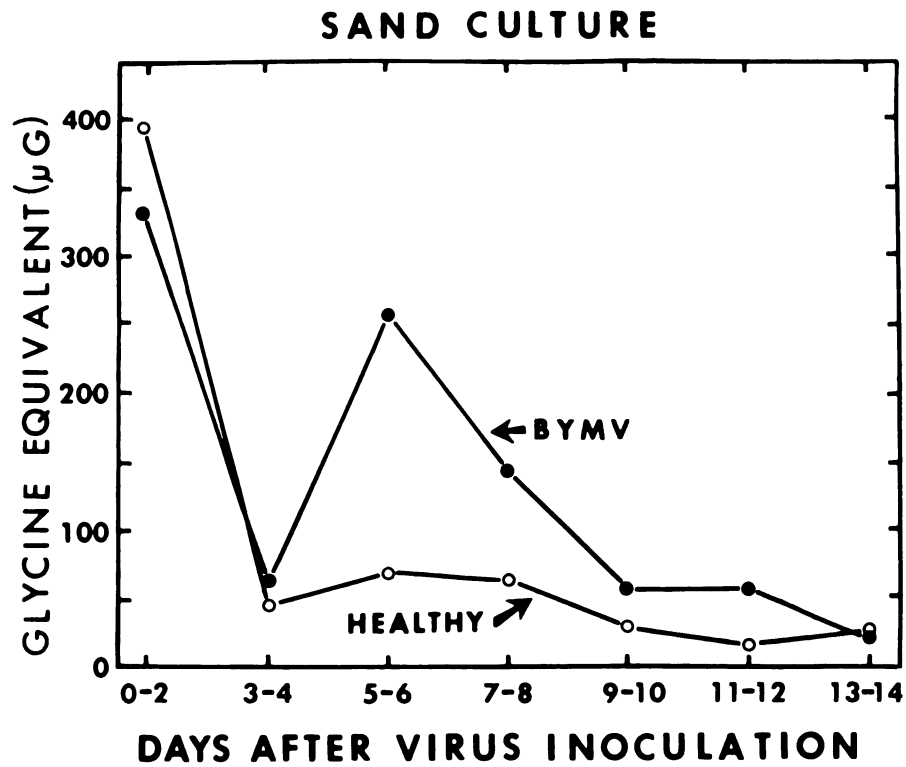


Figure 10. Effect of BYMV-infection on amounts of amino acids exuded by roots of pea plants growing in sand culture. Amino acid content was determined at 2 day intervals with ninhydrin reagent. Similar results were obtained in another test.

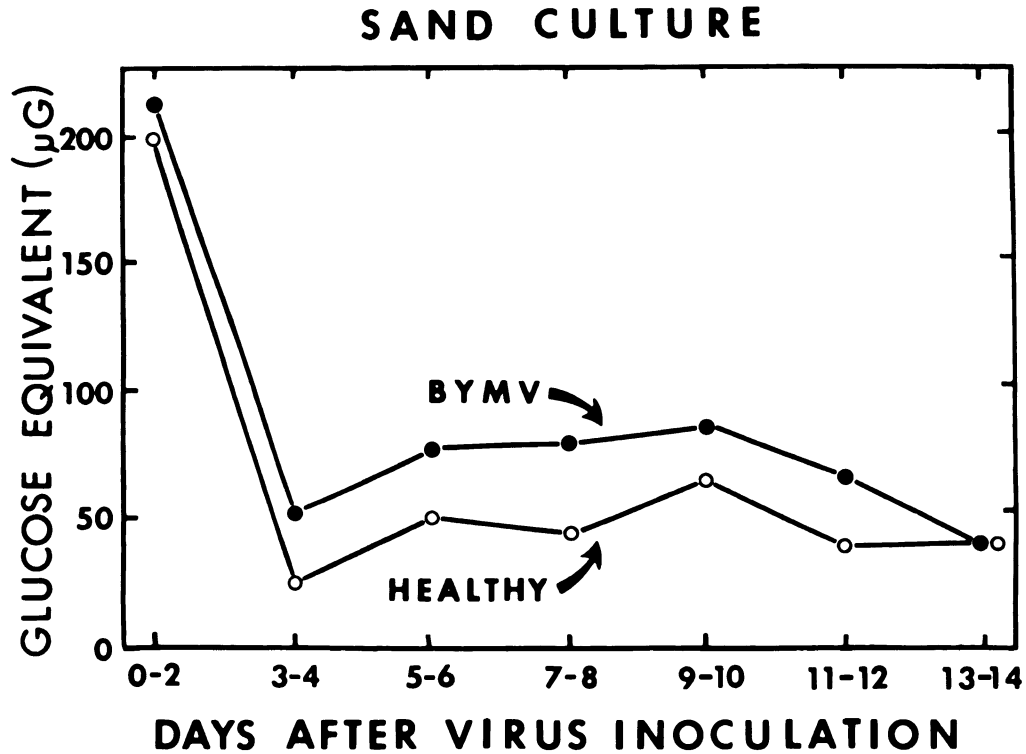


Figure 11. Effect of BYMV-infection on amount of carbohydrates exuded by roots of pea plants growing in sand cultures. Carbohydrate content was determined at 2 day intervals using a modified Dreywood's anthrone reagent. Similar results were obtained in another test.

and nucleotides occurred during this period. However, the relative increase in amount of these compounds released was never as great as for the amino compounds. Similar results were observed in another test.

Increased root exudation induced by PMV infection began 6-8 days after virus inoculation. Roots of BYMV-infected or PMV-infected plants released 4-5 μg of protein each day, the same as that released by healthy plants (5% level). A rather large quantity of all substances was released by both healthy and virus-infected plants 0-2 days after transplanting. This can be attributed to the shock of transplanting into acid washed sand since it did not occur with plants grown in water cultures.

Roots of healthy Miragreen peas growing in silica sand exuded approximately 25 μg of both carbohydrates and amino compounds per plant each day. During the period between 4 and 14 days after BYMV infection, the average increase in amount of nucleotides, carbohydrates and amino compounds was 22%, 55% and 137 %, respectively. Similar results were obtained in another tests using BYMV infection.

Although the actual quantity of substances exuded by roots differed in sand and water cultures, the change in root exudation induced by virus infection was similar in

both systems. The quantity of carbohydrates released per plant was the same in both water and sand cultures. Also, the periods of time during which increased exudation from roots of virus-infected plants occurred were the same. However, the quantity of amino compounds released per healthy plant in water culture was approximately 33% that of sand culture. Possibly, this difference resulted from lower light intensity or to increased utilization of nitrogenous compounds by microorganisms in water cultures. Total amino compounds in the exudate increased more than did total carbohydrates or nucleotides in both water and sand cultures.

Relationship between exudation period and disease:

If the increased root rot in virus-infected peas is a result of increased nutrients in the rhizosphere of virus-infected plants, the increased root rot should occur only during the period of increased exudation.

Ten Miragreen seeds were planted in a mixture of equal parts ground peat and sand in 4 in. plastic pots at 3 day intervals. When seedlings were 8 days old, the plants in half the pots were inoculated with BYMV. At the time of virus inoculation in the last-planted series of pots, plants in all pots were inoculated with a 20 ml suspension of F. solani conidia (10^6 /ml). Thus, the intervals established

between virus and fungus inoculations were 0, 3, 6, 9, 12, and 15 days. Disease severity was determined 17 days later. In another experiment, plants sown at the same time were inoculated with BYMV at different intervals of time. Plants were equally old at time of fungus inoculation. The previous intervals between virus and fungus inoculation were repeated.

The increased root rot induced by virus infection occurred only when fungus infection followed virus infection by 9 days or less (Figure 12). After this time, root rot severity was the same in virus + fungus-infected peas and in fungus-only-infected controls. Similar results were obtained in 2 additional tests using BYMV.

Root washing experiments were designed to determine whether the correlation between increased root exudation and increased root rot could be extended to the early stages of virus infection before increased root exudation was evident. Eight-day old Miragreen seedlings growing in silica sand were inoculated by pipetting a 10 ml suspension of F. solani conidia (10^6 /ml) on the sand surface. Plants in half the pots were simultaneously inoculated with BYMV. Plants from the virus + fungus treatment and fungus-only controls were removed from the pots immediately after inoculation, and at 3 and 6 day later, then washed thoroughly under running tap

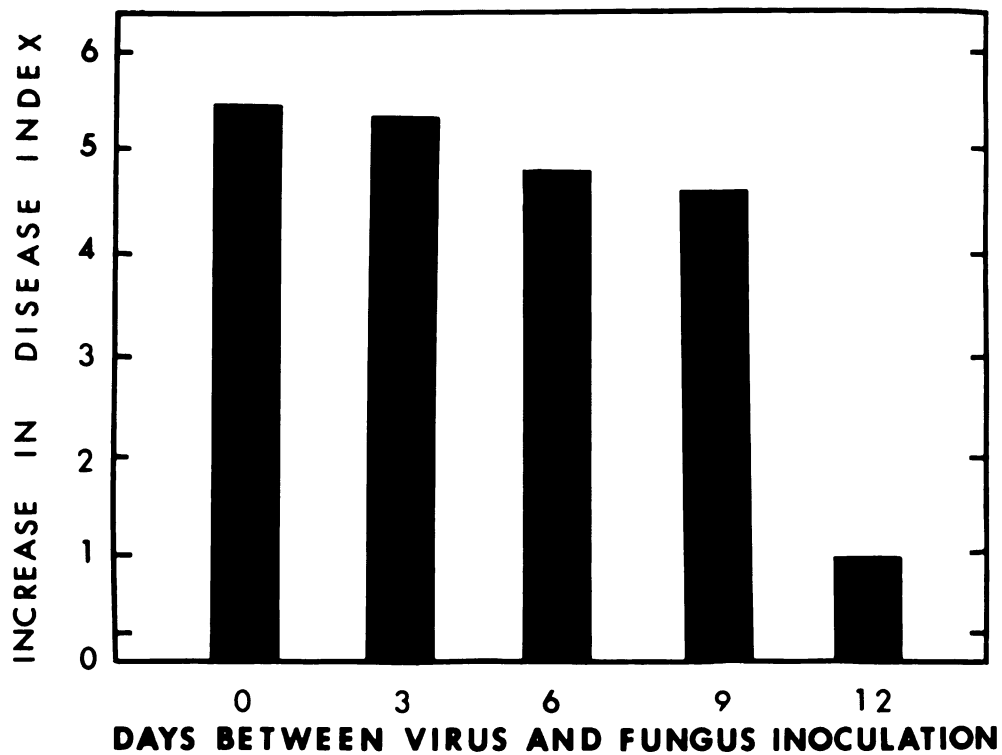


Figure 12. Relation between root rot severity and time interval between inoculation of Miragreen peas with BYMV and *Fusarium solani*. Values are expressed as the increase in disease index of fungus + virus-infected plants over fungus-infected plants of the same age. The increased root rot induced by virus infection occurred only when fungus infection followed virus infection by 9 days or less. Disease severity was determined on a scale of increasing severity from 0-9.

water to remove adhering spores and mycelia. The plants were replanted in non-infested silica sand. Disease severity was determined 17 days later. In another experiment, plants sown at the same time were inoculated with BYMV at different intervals of time. Immediately after the last series of plants was inoculated with BYMV, a 10 ml conidial suspension (10^6 /ml) of F. solani was pipetted on the sand surface. Three days later, all plants were removed from the pots, roots were washed thoroughly under running tap water, and plants were replanted in non-infested silica sand. Disease severity was determined 21 days later.

The increased root rot did not occur if the fungus was removed before the fourth day after virus inoculation, when increased exudation was known to first occur (Figure 13). However, if the fungus was present for an additional 1-2 days after increased exudation began, i.e., through 6 days after virus inoculation, increased root rot did occur (Table 3). Thus, a good correlation exists between the time increased exudation from virus-infected plants first occurs and the time when virus-infected plants show increased root rot.

These data indicate that increased root rot induced by virus infection in peas occurred only during the period of increased exudation from roots of virus-infected peas.

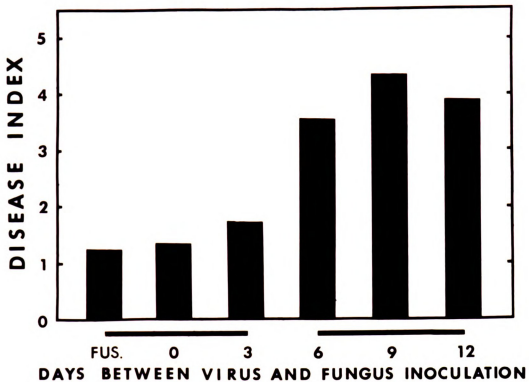


Figure 13. Relation between root rot severity and time interval between inoculation of Miragreen peas with BYMV and Fusarium solani. Plants sown at the same time were inoculated with BYMV at different intervals of time. When the last series of plants was inoculated with BYMV, all plants were inoculated with F. solani. Three days later plants were removed from pots, roots were washed, and plants were replanted in non-infested sand. Fusarium-only-inoculated plants (Fus.) served as controls. Disease severity was determined on a scale of increasing severity from 0-9. The increased root rot did not occur if the fungus was removed before the 6th day after virus inoculation, when increased exudation was known to first occur. Bars joined by a common line did not differ (5% level).

Table 3. Relationship between Fusarium root rot severity and the increase in root exudation beginning 4-6 days after virus inoculation^a

Time of fungal removal from root surface	Disease indices for plants infected with indicated pathogens ^b	
	Fungus only	Fungus +BYMV
0 days	1.3	1.5
3 days	2.0	2.0
6 days	4.3	8.0

^aPlants were simultaneously inoculated with virus and fungus. At the indicated intervals plants were uprooted, roots were washed, then transplanted into non-infested sand.

^bDisease indices were based on a scale of increasing severity from 0-9. Each figure is the mean of 3 pots, each with 10 plants. The only increase in disease index (5% level) with virus infection occurred at 6 days.

Biological activity of root exudates: One indication of an increased release of nutrient substances from roots of virus-infected as compared with healthy plants would be the amount of bacterial growth in water cultures of plants. Dilution plate counts made daily from such culture solutions generally showed a 2-3 fold increase in number of bacteria associated with BYMV or PMV infection. This increase in bacteria became evident 6-8 days after inoculation and continued until the experiment was terminated 16 days after virus inoculation. Similar results were obtained in 2 additional tests.

The effect of diluted root exudates on the growth rate of germ tubes of F. solani f. pisi was measured. Concentrated leachate from roots of BYMV-infected and healthy plants grown in sand was serially diluted to 10^{-6} the original concentration. Germ tubes of F. solani grew 32% longer in 12 hr in exudates from roots of virus-infected plants than in exudates from roots of healthy plants (Table 4). Thus, the exudate from virus-infected plants contains more growth supporting substances than exudates from healthy plants. Similar results were obtained in another test.

The germination of Helminthosporium victoriae conidia was observed on natural soil adjacent to roots of both healthy

Table 4. The effect of root exudates from healthy and virus-infected plants on growth of F. solani f. pisi germ tubes^a

Test	Germ tube lengths, μ ^b	
	Healthy plants	BYMV-infected plants
1	280	379
2	242	312
3	252	318

^aExudates from plants grown in silica sand were diluted to 10^{-6} original concentration. Duplicate plates were used in each test, and germ tube lengths of 20 spores were measured per plate.

^bGerm tubes grew longer in root exudates from virus-infected plants (5% level).

and virus-infected plants. Roots of 24-day-old plants infected with BYMV for 4, 8, 12 and 16 days were compared with healthy roots in their influence upon conidial germination over a 4-7 cm distance from the roots (Figure 14). Roots of plants infected with BYMV for only 4 days stimulated germination of conidia to the same extent as did healthy plants. When plants were used 8 days after virus inoculation, germination of conidia increased over that observed on soil adjacent to healthy controls. Moreover, the stimulation extended over a 3 cm distance from the roots. The highest germination of conidia and the greatest distance of stimulation occurred with plants 12 days after virus inoculation. At 16 days after virus inoculation, germination and distance of stimulation were markedly reduced. The periods of greatest stimulation of conidial germination coincided with the period of increased root exudation from virus-infected plants. Similar results were obtained in 3 additional tests using BYMV.

These data indicate that virus infection may result in an increased stimulation of fungal germination and growth over a longer distance from the root surface as compared with healthy plants. The actual distance over which stimulation was observed in these tests may be an artifact, however, resulting from the continual moisture film produced by

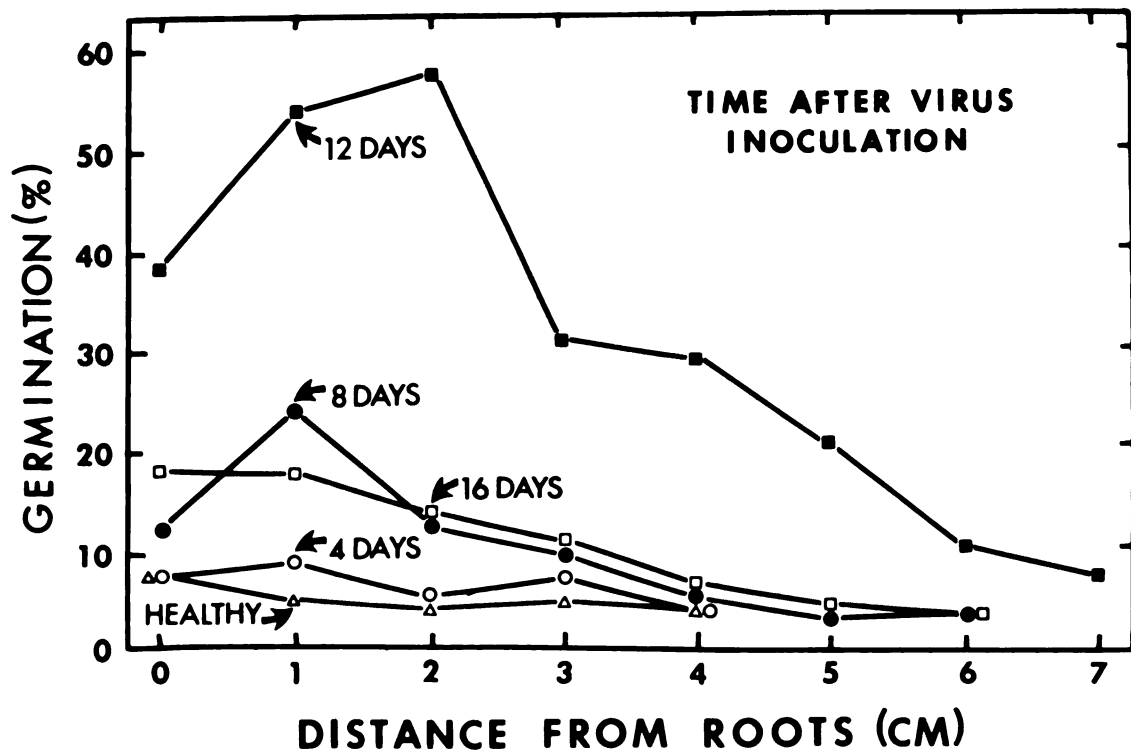


Figure 14. Germination of *Helminthosporium victoriae* conidia on soil adjacent to roots of healthy and virus-infected plants. Roots of plants infected with BYMV for 4, 8, 12 and 16 days were contained within a cellophane membrane in petri dishes. Soil was packed around the roots, and conidia were applied to the smoothed soil surface. Highest germination of conidia and greatest distance of stimulation coincided with the period of increased root exudation from virus-infected plants. Similar results were obtained in 3 additional tests.

smoothing the soil surface.

Chromatographic analysis: Amino acids: Portions of the concentrated cationic fraction collected through a 6-12 day period after virus infection were chromatographed. Based on the total quantity of ninhydrin positive compounds in the root exudate, equivalent quantities of the amino acid fractions of healthy and BYMV-infected root exudate solutions were applied to the paper.

Seventeen ninhydrin-positive spots appeared on chromatograms of virus-infected root exudates while 14 spots were detected on chromatograms of healthy root exudates. Ten of these were identified on each and the amounts determined (Table 5). The unidentified spots on the chromatograms were estimated to represent less than 5% of the total ninhydrin positive substances present. All amino acids were released in greater quantities from roots of virus-infected plants than from healthy roots. The total increase was 3-4 fold that of healthy plant exudates. The concentration of alanine increased the most in relation to other amino acids. Alanine was 4% of the total amino acids in healthy plant exudates but increased to 21% of the total in virus-infected plant exudate. Thus, the absolute amount of alanine increased 20-fold in root exudates from virus-infected plants. The

Table 5. Amino acid composition of exudates from roots of healthy and virus-infected Miragreen peas grown in silica sand^a

Amino acids	Healthy plants		BYMV-infected plants	
	ug/plant/ day ^b	% of total	ug/plant/ day ^b	% of total
asparagine	1.3	12	2.9	7
aspartic acid	0.2	1	0.4	1
glutamic acid	0.2	1	0.4	1
glutamine	0.6	4	1.5	4
serine	0.2	1	0.7	2
tryptophane	0.8	7	3.6	9
γ-amino butyric acid	3.8	35	5.8	15
alanine	0.6	4	8.4	21
threonine	3.8	35	13.1	33
phenylalanine	0.0	0	2.9	7

^aAmino acids were detected with 0.5% ethanolic ninhydrin, spots were cut from developed chromatograms, eluted in 50% ethanol and compared with a standard curve for each amino acid prepared on paper in a similar manner.

^bEach value represents the average daily amount exuded by 15 plants during the period 6-12 days after virus infection. Similar results were obtained using exudates from another experiment.

respective relative concentrations of γ -amino butyric acid and asparagine were 35% and 12% of the total in healthy plant exudates and 15% and 7% in virus-infected plant exudate. However, the total amounts of γ -amino butyric acid and asparagine in root exudates from virus-infected plants were increased by 53% and 123%, respectively. Threonine was the most prominent amino acid in root exudates from BYMV-infected plants. Although its concentration in relation to other amino acids was the same in both exudates, the increase in the exudate from virus-infected plants was 263% as compared with healthy root exudates. A striking difference was the presence of phenylalanine in root exudates from virus-infected plants and its absence in root exudates from healthy plants. The results show that, in addition to a general increase in exudation of all amino acids from roots, selective increases in specific amino acids also occur. Similar results were obtained with exudates from another experiment.

Amino acid content of root tissue was examined to determine if changes in exudation of these compounds reflected changes in the free amino acid pool in the tissue resulting from virus infection. Root tissues from virus-infected and healthy pea were extracted with distilled water on hot 80% ethanol. Tissue extract was prepared and chromatographed by

the same method used for root exudates. Analyses of total carbohydrates and amino acids indicated no differences (5% level) in total soluble pools of either class of compounds over a 20 day infection period (Table 6). Similar results were obtained in 3 tests. Virus-infected and healthy Mira-green plants both contained approximately 2.4 mg free amino acid and 7.1 mg carbohydrate per root. In this test, amounts of individual amino acids were similar in virus-infected and healthy tissue. Phenylalanine was present in root tissues of both virus-infected and healthy pea (Table 7). The 3-4 fold increase in amount of amino acids exuded from roots of virus-infected plants cannot be explained by changes in total quantities or in specific amino acids within root tissue.

Organic acids: Four major groups of organic acids were separated (Table 8). The only detectable increase in organic acids released from virus-infected plants occurred among those compounds having an Rf value of 0.02. This group includes gluconic, galacturonic and glucosaccharic acids (7). No further attempt was made to identify these compounds. Samples of the concentrated solutions were titrated with 0.0144 N NaOH using phenolphthalein as an indicator (60). The acid equivalent in root exudate from virus-infected plants was 140% that of healthy exudate. Similar results were

Table 6. Total carbohydrates and free amino acid pools in healthy and BYMV-infected Miragreen pea root tissue

Infected with virus for	mg/g/pea root tissue ^a	
	Amino acids	Carbohydrates
4 days	2.4	6.0
8 days	2.1	7.7
12 days	2.9	10.2
16 days	2.5	6.5
20 days	2.3	6.0
Healthy	2.3	6.3

^aValues are the average for 10 plants. Amino acid content was determined by the ninhydrin reagent, carbohydrate content by the anthrone reagent. The quantities of amino acids or carbohydrates in root tissues of healthy plants did not differ from those in plants infected with BYMV (5% level). Similar results were obtained in 2 additional tests.

Table 7. Free amino acid pool in 20-day-old root tissue of healthy and virus-infected Miragreen pea after virus inoculation^a

Amino acids	Healthy plants		BYMV-infected plants	
	ug/plant/ day ^b	% of total	ug/plant/ day ^b	% of total
asparagine	2.3	30	2.3	26
aspartic acid	0.2	2	0.3	4
glutamic acid	0.1	1	0.2	2
serine	0.1	1	0.1	1
tryptophane	0.3	5	0.4	5
γ-amino butyric acid	0.1	1	0.1	1
alanine	0.1	1	0.2	2
threonine	2.8	41	4.5	52
phenylalanine	0.3	4	0.6	6

^aAmino acids were extracted by grinding 2 g root tissue in a mortar with 10 ml of 80% hot ethanol. Amino acids were detected with 0.5% ethanolic ninhydrin, spots were cut from developed chromatograms, eluted in 50% ethanol and compared with a standard curve for each amino acid prepared in a similar manner.

^bValues are the average for 10 plants. The average plant root weight was 3 grams.

Table 8. Organic acids in exudates from roots of healthy and virus-infected Miragreen peas grown in silica sand

Rf value	Possible compounds	Diameter of spot on chromatogram, mm ^a	
		Healthy plants	BYMV-infected plants
.02	gluconic, glucosaccharie and galacturonic acids	12	18
.45	glycolic, diglycolic, and ketoglutaric acids	15	15
.69	pyruvic, aconitic, and citraconic acids	17	17
.93	furoic, mesaconic, and sorbic acids	8	8

^aEach value represents the average content from 2.3 plants each day during the period 6-12 days after virus inoculation. Similar results were obtained in 2 additional tests.

in 2 additional tests and with root exudates from PMV-infected pea.

Reducing sugars: When root exudates from 16-20 day old healthy or virus-infected Miragreen seedlings were chromatographed, reducing sugars were either not present or were present in such low concentrations that they could seldom be detected. Results with Glucostat reagent indicated an average release of $0.88\text{ }\mu\text{g}$ of glucose by healthy plants each day during this period, whereas roots of virus-infected plants exuded an average of $1.76\text{ }\mu\text{g}$ per plant each day during this period.

The effect of virus infection on phosphate ion uptake: The similarity in the size and composition of the free amino acid pools of healthy and virus-infected plants indicated that the increased release of amino acids from roots of virus-infected plants was not due to alterations in the concentrations of these substances within the root tissues. The possibility that the increased exudation was related to increased permeability of cell membranes to small molecular compounds was investigated.

Roots of Miragreen peas infected with BYMV for 12 days accumulated two times more radioactivity than did healthy roots, when both were immersed in a buffer solution containing

$\text{H}_3^{32}\text{PO}_4$ for 2 hr (Table 9). In two other experiments, plants exposed to $^{32}\text{PO}_4$ for 1 hr accumulated 24-26% more radioactivity than did healthy roots. Two explanations for increased uptake were considered: 1) Permeability of plasma membranes was increased, or 2) active uptake of PO_4 was increased due to virus infection. Pretreatment of roots with 2,4-dinitrophenol (10^{-4}M), an uncoupler of oxidation from phosphorylation, reduced accumulation of $^{32}\text{PO}_4$ in both virus-infected and healthy roots by 11-14% as compared with buffer control. Since the reduction in each was proportional, no selective decrease occurred in the case of virus-infected plants (Table 10). These data indicate that PO_4 accumulation by pea roots is not energy dependent and suggests that the increased uptake of phosphate by roots of virus-infected plants was due to increased permeability of plasma membranes. Probably, the increased leakage of substances from roots of virus-infected plants is also due to increased membrane permeability.

Effect of glucose, amino acids and organic acids on root rot: Increased quantities of carbohydrates, organic acids and amino acids were released by roots of virus-infected plants as compared with those of healthy plants. When root exudates containing these substances were added to plants

Table 9. The effect of virus infection on uptake of ^{32}P by root tissue of Miragreen pea^a

Treatment	CPM/g dry weight of roots (x 1,000) ^b
Virus-infected plants	2,182
Non-virus-infected plants	1,064

^aRoots were exposed to ^{32}P in 10^{-3} M PO_4 buffer (pH 7.0) for 2 hr. Plants were inoculated 12 days previously with BYMV.

^bValues are the mean of 3 replicates. Roots of virus-infected plants accumulated more radioactive ^{32}P (5% level) than did healthy roots. Similar results were obtained in 2 additional tests.

Table 10. The effect of 2,4-dinitrophenol on uptake of ^{32}P by root tissues of virus-infected and healthy Miragreen pea^a

Pretreatment	CPM/g wet weight of roots (x 1,000) ^b	
	Healthy plants	BYMV-infected plants
PO_4 buffer	36	44
DNP in PO_4 buffer	31	39

^aPlants inoculated 8 days previously with BYMV were placed in 10^{-3} M PO_4 buffer (pH 7.0) containing 2,4-dinitrophenol (10^{-4} M) for 2 hr or in buffer alone for 2 hr before a 1 hr exposure to ^{32}P in 10^{-3} M PO_4 buffer.

^bValues are the mean of 2 replicates of 3 plants each. Roots of virus-infected plants accumulated more radioactivity (5% level) than did healthy roots.

inoculated with F. solani or A. euteiches, increased root rot occurred. Therefore, the prominent compounds occurring in the root exudate from virus-infected plants were used to prepare a simulated root exudate which was applied to inoculated plants. Miragreen seedlings growing in moist silica sand were inoculated with a 10 ml suspension of F. solani conidia (10^6 /ml). Daily for 8 days after inoculation, each pot received a 10 ml water solution containing: 6 mg glucose; 3 mg galacturonic acid and 3 mg glucuronic acid; 3 mg of each of the following amino acids: asparagine, alanine, γ -amino butyric acid, tryptophane, threonine, and phenylalanine; and a combination of all these compounds. Thus, each pot containing 10 plants received approximately the amount of each substance released by 40 virus-infected plants (based on analysis of exudates from plants grown in sand).

Disease severity was the same (5% level) in plants receiving glucose or organic acids as in plants receiving only water (Figure 15). Disease severity of plants receiving amino acids or a combination of all compounds was 2-fold greater than plants receiving only water (Figure 16). Similar results were obtained in 2 additional tests. Substitution of glutamine for phenylalanine did not alter

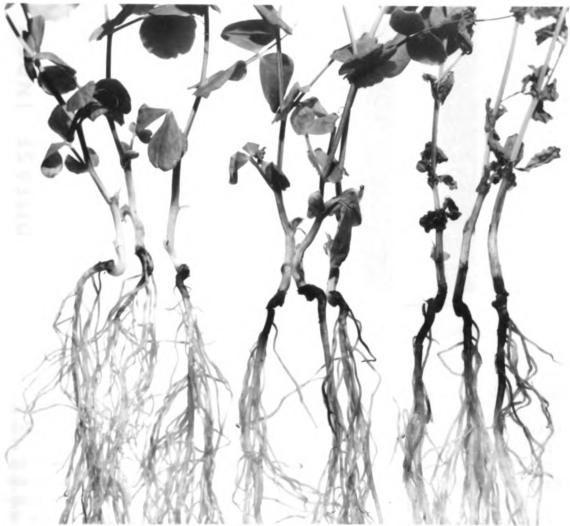


Figure 15. Fusarium root rot development on Miragreen peas receiving glucose, organic acid or amino acid amendments. Daily for 8 days after fungus inoculation, 6 mg of glucose (left), 3 mg of each of 2 selected organic acids (center), and 3 mg of each of 6 amino acids (right) were pipetted in 10 ml of water, individually or in combination on 10 plants growing in silica sand. Symptoms on plants receiving only water did not differ from those on plants receiving glucose.

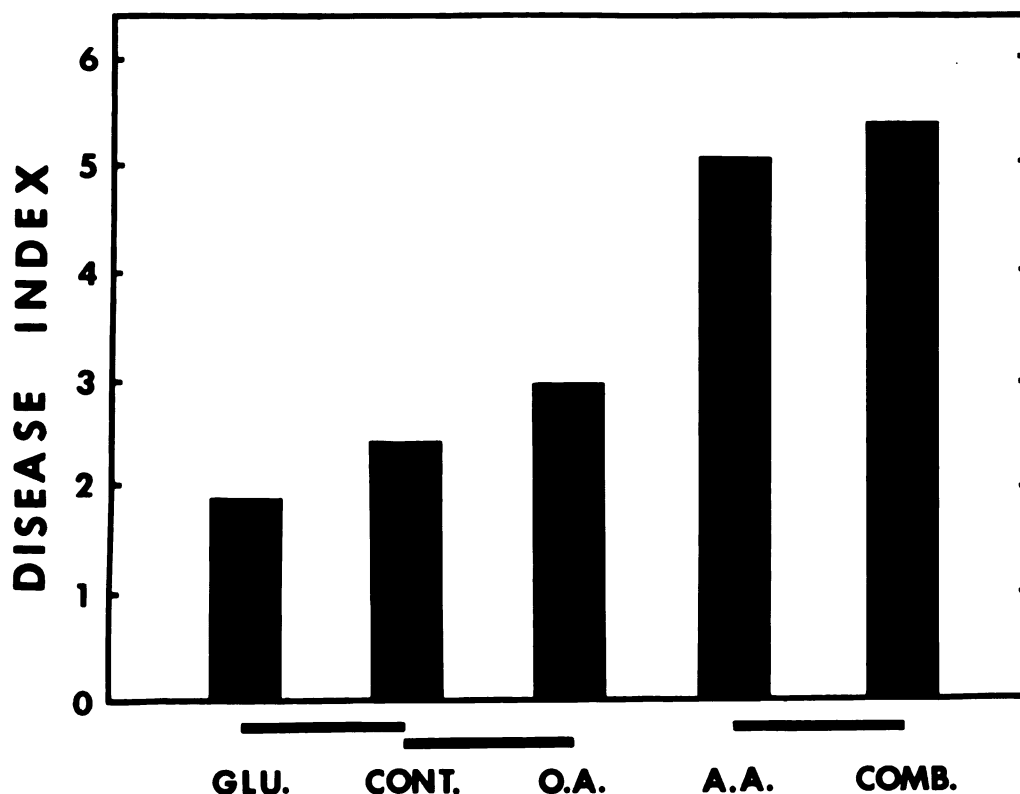


Figure 16. Effect of glucose, amino acids and organic acids on development of Fusarium root rot of peas. Daily for 8 days after fungus inoculation, 6 mg of glucose (Glu.), 3 mg of each of 2 selected organic acids (O.A.), and 3 mg of each of 6 amino acids (A.A.) were pipetted in 10 ml of water, individually or in combination (Comb.) on 10 plants growing in silica sand. Similar results were obtained in 2 additional tests. Bars joined by a common line did not differ (5% level). Disease severity was determined on a scale of increasing severity from 0-9.

the stimulatory effect of this group of amino acids on root rot severity; therefore, phenylalanine was not a critical factor in increased inoculum potential. Thus, the increase in inoculum potential of F. solani can be explained on the basis of a general increase in amino acids exuded from roots of virus-infected plants.

Effect of fungus infection of plants on the virus:

In view of the striking effect of virus infection on development of fungal root disease of pea, it was of interest to determine whether fungus infection would in any way alter virus infection or virus content in the plant.

Infection with F. solani f. pisi caused an early increase in infectivity of BYMV or PMV in pea leaf sap. A 2-3 fold increase in local lesions was noted using BYMV + F. solani-infected leaf sap beginning 5 days after inoculation. Beyond 12 days from initial inoculation, however, no differences in virus titer could be determined. Infection with F. solani resulted in a 2-3 fold increase in PMV infectivity between the third and fourth day after inoculation. Thereafter, leaf sap from virus-infected or fungus + virus-infected plants was equally infective.

Infection of pea with F. oxysporum f. pisi failed to influence the infectivity of virus in pea leaf sap. Moreover,

observations from a single test indicate that BYMV infection had no effect on Fusarium wilt disease.

Role of root exudation in varietal resistance to root rot: Data presented in this research indicate that severity of root rot in pea is increased when the pathogen is exposed to increased amounts of root exudates before or during infection. This concept of inoculum potential as a factor in root rot severity was extended to 2 experimental pea varieties being used in a program of breeding for disease resistance (Lockwood and Beute, unpublished results). In greenhouse tests, variety 333 has moderate resistance to Aphanomyces root rot, and variety GW-4 possesses moderate resistance to both Aphanomyces and Fusarium root rots. Root exudates from Miragreen pea, which is susceptible to both diseases, were compared with those of the 2 experimental varieties. Plants were grown in glass-distilled water.

Both experimental varieties released fewer electrolytes than did Miragreen (Figure 17). Total amino acids in culture solutions of the variety GW-4 were only 53%, and those of variety 333, 75% of those from Miragreen. However, both resistant varieties released 24-31% more carbohydrates than did Miragreen. Thus, if nutrients in the rhizosphere are a factor in the relative susceptibility of these experimental

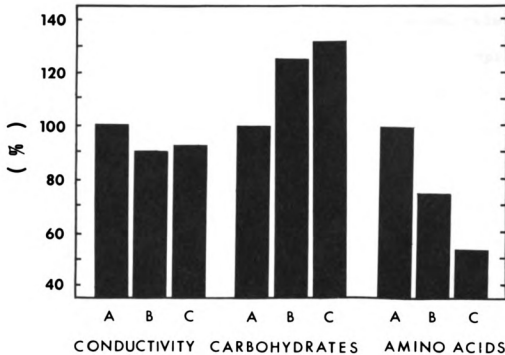


Figure 17. Relative exudation of electrolytes, carbohydrates and amino acids by healthy A) Miragreen pea, which is susceptible to both Aphanomyces and Fusarium root rot, B) Variety 333, resistant to Aphanomyces root rot, and C) Variety GW-4, resistant to both pathogens. Plants were grown in water culture. Values are the average daily exudation over a 16 day period expressed as a percentage of Miragreen.

pea varieties to root rot disease, the decreased release of amino compounds, but not carbohydrates, would be implicated in decreasing the inoculum potential in the root area. The specific compounds present in these exudates were not determined.

The effectiveness of root leachings from each of the 3 pea varieties on Aphanomyces or Fusarium root rot was determined. Each pot containing A. euteiches-infected Miragreen seedlings received root leachings from 20 plants of one of the 3 pea varieties, while each pot of F. solani-infected Miragreen seedlings received root leachings from 30 plants of one of the 3 pea varieties.

The disease index for A. euteiches in Miragreen seedlings receiving root leachings from healthy Miragreen seedlings was more than double that of seedlings receiving no root leachings (Figure 18). The disease index for A. euteiches in Miragreen seedlings receiving root leachings from either variety 333 or GW-4 did not increase (5% level) above plants receiving no leachings.

Similarly, the disease index for F. solani in Miragreen seedlings receiving root leachings from healthy Miragreen plants was almost double that of seedlings receiving no leachings (Figure 18). The disease index for F. solani in Miragreen

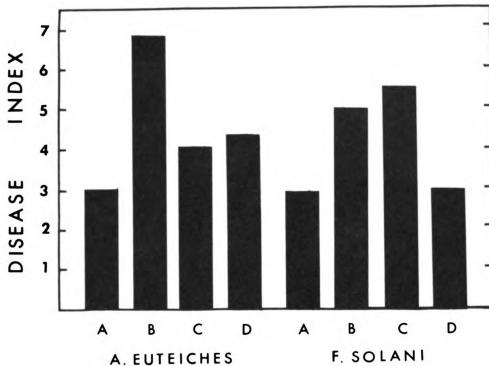


Figure 18. Effect of root leachates from 3 pea varieties with different resistances to Aphanomyces or Fusarium root rot on root rot severity in Miragreen peas. A) Plants received leachates from sand. B) Plants received root leachates from Miragreen plants. C) Plants received root leachates from Aphanomyces resistant variety 333. D) Plants received root leachates from variety GW-4, resistant to both pathogens. Fusarium root rot was increased (5% level) by leachates from both Miragreen plants (B) and Aphanomyces resistant plants (C). Aphanomyces root rot was increased only by leachates from Miragreen plants (B). Disease severity was determined on a scale of increasing severity from 0-9.

seedlings receiving leachings from healthy GW-4 plants was not increased (5% level) above fungus-infected plants receiving no leachings. However, F. solani-infected Miragreen seedlings, receiving root leachings from variety 333 which is resistant to A. euteiches only, were as severely diseased as plants receiving root leachings from Miragreen seedlings.

The levels of resistance observed in the experimental pea varieties may be a reflection of the influence of root exudates on inoculum potential in the rhizosphere. The effect of root exudates from the 3 pea varieties on root rot severity are compatible with this interpretation.

The fact that root exudates from Aphanomyces resistant plants failed to increase root rot (5% level) on Aphanomyces-infected Miragreen plants but did increase root rot on Fusarium-infected plants indicates that qualitative as well as quantitative differences in root exudates may be responsible for changes in inoculum potential. Exudates from resistant plants never decreased root rot when used as amendments to fungus-infected plants, indicating that root exudates are not acting in a defensive role.

DISCUSSION

Little is known about the metabolic interactions occurring when severity of a fungal disease is altered by virus infection of the host. Several suggestions have been made. Plant constituents essential for pathogenic development may have increased or decreased (2, 8, 9, 44, 68). Increased resistance to powdery mildew in leaf roll-infected grapes has been suggested as due to increased osmotic pressure of the host cells (19). Decreased resistance to late blight in potato infected with leaf roll virus has been attributed to a more favorable microclimate for infection in leaves malformed due to virus infection. However, no evidence has been presented to support these suggestions.

The virus-induced increased root rot observed in pea (12, 13, 64) apparently is not due to changes in inherent susceptibility of pea roots. This is indicated by several lines of evidence: 1) the typical virus-induced increase in pea root rot was not observed when all inoculum exterior to the root surface was removed before fungus-infected plants were inoculated with the virus. 2) Little or no

alternation in susceptibility was evident on the basis of lesion length and severity when F. solani conidia were injected directly into the hypocotyl. 3) Removal of exudates from healthy and virus-infected plants by periodic leachings of their roots resulted in plants being equally susceptible to root rot. These results indicated that the critical factor in the mechanism of increased root rot was operating exterior to the root surface, i.e., in the rhizosphere.

One of the early effects observed in some host-parasite interactions involving fungi is the enhanced release of intracellular materials from host cells. This effect may either be the result of physical disruption (29, 57), or the pathogen may alter cells at a considerable distance in advance of its actual invasion (11, 55). Wood and Braun reported that crown-gall tissue took up more exogenous solutes than did healthy tissues (67). They concluded that permeability of one or more of the membrane systems of the cell was increased in some way by the bacterium, Agrobacterium tumefaciens.

Virus infection may also enhance the release of intracellular materials from cells. Infection of Tabasco pepper by tobacco etch virus resulted in a marked release of electrolytes from roots of these plants (16). Also,

changes in rhizosphere microflora of Dolichos lablab infected with dolichos enation mosaic virus suggest changes in root exudation (31, 54).

In the present study, analysis of the nature and quantity of substances in root exudates, as well as the time of increased exudation from roots of BYMV-infected plants, revealed that carbohydrates, amino acids, organic acids and nucleotides were exuded in larger amounts from roots of BYMV-infected plants than from healthy plants. Increased release began 4-6 days after virus inoculation and continued until 14-16 days after inoculation. Although some amino acids increased more than did others, the increased release of amino acids from roots was the most pronounced effect observed in virus-infected plants.

The amount of amino acids released by roots of virus-infected peas was 2-3 fold that released by healthy roots. By contrast, the quantity of exudates released by virus-infected roots may have a direct effect on inoculum potential. Indeed, addition of root exudates from virus-infected plants to plants inoculated with A. euteiches or F. solani resulted in more severe root rot than did addition of root exudates from healthy plants. Moreover, increased root rot induced by virus infection in peas occurred only during the period

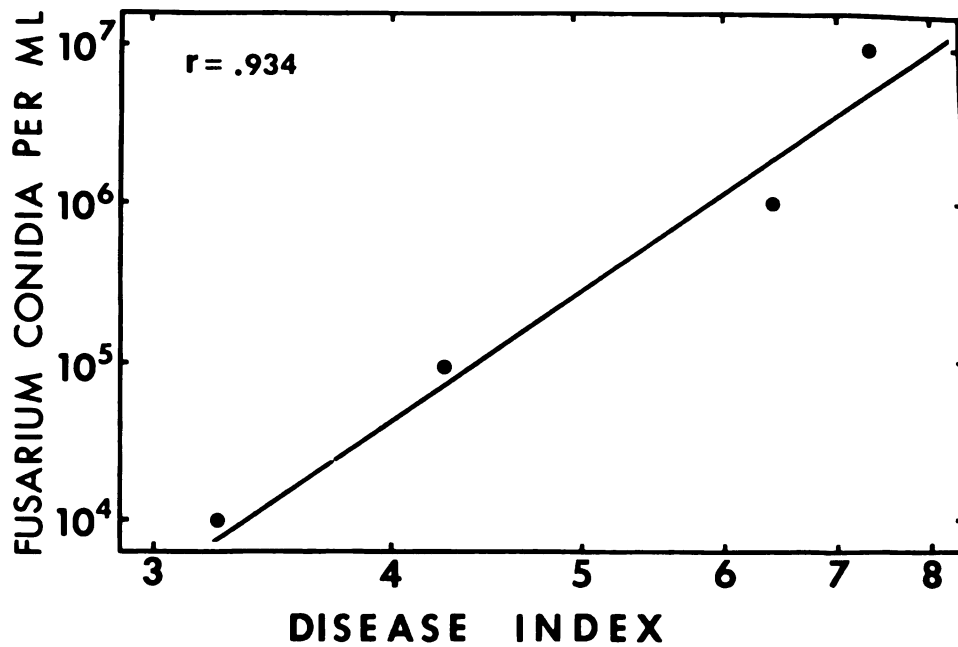


Figure 19. Severity of Fusarium root rot as a function of inoculum density. Four pots, each containing 10 Miragreen plants growing in silica sand, were used for each inoculum concentration. A 10 ml conidial suspension was pipetted on the sand surface. Disease severity was determined on a scale of increasing severity from 0-9.

of increased exudation from roots of virus-infected peas.

Chromatographic analyses of amino acids released from virus-infected roots showed a general increase of most compounds identified. The 3-4 fold increase in exudation of amino compounds could not be explained by increased quantities of these compounds within virus-infected root tissue, since concentration of individual amino acids within roots were changed very little by virus infection. Specific changes in amino acid release, such as the presence of phenylalanine in virus-infected root exudate and its absence in healthy root exudates, suggest alternation of membrane permeability of these root cells. More $^{32}\text{PO}_4$ was taken up by roots of virus-infected plants than by healthy roots. This increased uptake was not affected by exposure to 2,4-dinitrophenol, indicating that permeability of the membrane, rather than active uptake by the membrane, was increased.

Although many substances were increased in root exudates from virus-infected pea plants, amino compounds showed the greatest increase. In tests with a synthetic root exudate added to F. solani-infected peas, amino acids as a group, but neither glucose nor organic acids, were able to increase disease. However, differences in

stimulatory capacity of root exudates observed in these studies cannot be interpreted in terms of defense by roots against pathogenic invasion, since exudates never decreased root rot below levels occurring with no additional exudate. Further work is needed before the role of exuded amino acids in resistance or susceptibility of these pea varieties can be fully clarified. Possibly, differential root exudation may play a role in quantitative resistance to nonspecialized pathogens, such as root and stalk rot fungi. If so, susceptible roots may inadvertently contribute to their own disease severity by their relative success or failure in retaining within their root tissue small molecular compounds which are stimulatory to potential pathogens in the rhizosphere. The fact that seed exudation has been shown to be a factor governing susceptibility of peas and beans to pre-emergence damping-off (14) supports this conjecture.

The mechanism of increased virus infectivity in plants infected with F. solani and BYMV or PMV was not studied. Possibly, the rates of translocation of BYMV and PMV are increased due to the physiological stress imposed by F. solani invasion. In systemic viral infections, invasion of newly formed healthy tissue must occur either by rapid translocation through the vascular system or by slow passage from cell to

cell through plasmodesmata. Virus movement and translocation into newly formed pea leaf tissue are known to be affected by host nutrition and temperature (38).

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