Association of Virus-Induced Changes in Laimosphere Microflora and Hypocotyl Exudation with Protection to Fusarium Stem Rot

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ABSTRACT

Microbial populations were 2- to 7-fold higher in soil surrounding (0-3 mm) hypocotyls (laimosphere) of squash mosaic virus-infected squash (*Cucurbita maxima*) plants than healthy ones, and virus infection stimulated an increase (approx. 40%) in hypocotyl exudation. However, hypocotyl penetration by *Fusarium solani* f. sp. *cucurbitae* and initial lesion development were not influenced by virus infection. Yet protection to Fusarium stem rot was induced by squash mosaic and was expressed if the inoculum was in soil. Protection was overcome at higher fungal inoculum levels. The influence of squash mosaic on the laimosphere microflora and the regulation of protection by *Fusarium* inoculum levels in soil supports evidence that virus-induced resistance to stem rot is expressed during the prepenetration phase of pathogenesis.

There were no differences in the rates of permeation of urea across hypocotyl cell membranes in healthy or squash mosaic virus-infected squash plants. Furthermore, virus infection had no influence on the rate of uptake of 3-*o*-methylglucose, 2-aminoisobutyrate, glucose, alanine, glutamate, leucine, glycolate and malate by hypocotyl tissues. Apparently changes in membrane permeability or the activity of certain membrane transport systems do not account for hypocotyl exudation increases associated with squash mosaic.

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Additional key words: permeability, membrane transport.

Modification of plant disease expression during multiple infections is a common phenomenon (4, 5, 6, 7). The influence of virus infection on fungal diseases is often conspicuous and has received considerable attention (3, 10, 14, 17). Virus infection frequently alters its host's susceptibility to fungal diseases either increasing or reducing their severity.

Since a wide variety of fungal diseases are influenced by virus infection, explanations for changes in susceptibility often have little in common. One frequently suggested basis for alterations in susceptibility concerns changes in the metabolite level of host tissues. Goheen and Schnathorst (7) reported that leaf roll of grapes increased the sugar content of leaves resulting in an osmotic pressure unfavorable for powdery mildew. In contrast, lettuce mosaic lowered the osmotic pressure of host cells resulting in conditions favorable for powdery mildew (18).

In work with a soil-borne fungus disease, Beute and Lockwood (2) observed that root exudation increased from pea mosaic virus and yellow bean mosaic virus-infected pea plants. They considered these changes responsible for the increased susceptibility to Fusarium root rot. Diaz et al. (3) showed that virus-infected squash plants survived longer than virus-free plants when they were inoculated with *Fusarium solani* (Mart.) Appel and Wr. f. sp. *cucurbitae* Snyd. and Hans. race 1 but that protection was reduced by increasing the inoculum level. Because *Fusarium* chlamydospore germination was reduced in the soil surrounding the subterranean hypocotyl of virus-infected squash, they suggested that changes in the laimosphere were responsible for a reduction of the inoculum potential of the stem rot pathogen.

This investigation examines further the influence of squash mosaic on susceptibility to *F. solani* f. sp. *cucurbitae*. The effect of virus infection on (i) microbial populations in the laimosphere, (ii) hypocotyl exudation, (iii) chlamydospore germination, and (iv) fungal penetration and lesion development are considered.

MATERIALS AND METHODS.—*Estimation of microbial population in the laimosphere.*—Squash (*Cucurbita maxima* Dcne 'Pink Banana') were grown in an agricultural sandy loam soil and maintained as previously described (3). Squash mosaic virus (SMV) (common strain) inoculations throughout this investigation were made by grinding infected leaf tissues in 1% KH_2PO_4 containing 5% Celite and rubbing the juice gently on cotyledons of 6- to 7-day-old plants. Microbe populations around the hypocotyl of 14-day-old healthy and virus-infected plants were estimated by dilution plate methods (12).

Protection in the pre-infection phase and effect of virus infection on chlamydospore germination and penetration.—Squash plants were grown in 15.2-cm (6-inch) diam pots containing U.C. mix (1) and inoculated with SMV. *Fusarium solani* f. sp. *cucurbitae* was single-spored twice a month and maintained on potato-dextrose agar under continuous light. From the surface of the 6-wk-old cultures conidia were suspended in 3 ml water, macroconidia counted in a hemacytometer and adjusted to the desired concn. One ml of each dilution of macroconidia (1×10^5 and 2×10^6 conidia/ml) was pipetted into the soil around the hypocotyl of 14-day-old healthy and SMV-infected squash plants. Plants were subirrigated with water twice a day. Evaluation of disease development was made three times during the first week and daily thereafter.

Chlamydospores of *F. solani* f. sp. *cucurbitae* were obtained by mixing 5 ml inoculum, 10^{10} conidia/ml, with

10 g of sandy loam soil (Salinas series, California, USA). To obtain a suspension of macroconidia in the inoculum, spores were carefully removed with a wire needle from the surface of the agar and suspended in water. A small beaker containing the mixture of soil and spore suspension was covered with aluminum foil and kept in darkness at room temp (approx. 20 C) for 4 wk. Chlamydospore concn was adjusted with 100 g soil.

After plants were removed from pots, soil was shaken from the roots and roots were washed gently with water. Hypocotyls were rinsed with sterile water and 0.1% chloramphenicol. Four healthy and virus-infected plants were placed in a tray containing Hoagland's solution and the roots were aerated with filtered air. Four water droplets containing either four to five chlamydospores or two to three macroconidia were placed with a micropipette on the surface of virus-infected and healthy plant hypocotyls. After 8, 16, 24, and 36 h, plants were removed and the epidermis was carefully stripped from the hypocotyls. Mycelial growth, penetration and initial lesion development of the fungus was observed microscopically after the hypocotyl epidermis was immersed in 0.1% 2(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride solution for 5 min at 30 C.

Collection and analysis of ^{14}C-labeled hypocotyl exudates.—Squash plants were grown in vermiculite in 10.2-cm (4-inch) diam pots in a constant environment chamber. Plants were illuminated for 14 h/day with 20-W fluorescent tubes and 100 W incandescent bulbs (17,200 lux). Daytime temp was maintained at 25 C with a 3 C reduction at night. Relative humidity ranged between 60 and 70%.

Six days after planting, seedlings were inoculated with SMV as previously described. Sixteen days after inoculation, 10 healthy and 10 infected plants were removed from pots and the vermiculite washed from the roots. Hypocotyls were washed with a 0.1% chloramphenicol solution. Hypocotyl exudates were collected on 0.2 × 10-cm filter paper strips which were carefully wound around the hypocotyls. The plants were replanted with hypocotyls exposed. The plants were placed in a circular air-tight Plexiglas box and returned to the growth chamber where constant light (17,200 lux) and temp (20 C) were maintained. Radioactive $^{14}CO_2$ was generated by adding 5 ml 50% lactic acid to a reaction vessel containing 4 ml of Ba $^{14}CO_3$ (specific activity 58.8 mCi/mmole). Circulation of $^{14}CO_2$ in the chamber was maintained by a battery operated fan. After 15 min, air was flushed through the chamber at a rate of 0.7 liter/min. Thirty-six h after the liberation of $^{14}CO_2$, the filter paper around the hypocotyl was removed with care, and the water-soluble, ^{14}C-labeled exudates were eluted with water, and then dried in a vacuum evaporator. Final volume was brought to 0.5 ml with 80% ethanol. Following collection of exudates, hypocotyls were extracted twice with 80% hot ethanol for 30 min. Radioactivity was determined in 10 μl aliquots by liquid scintillation (13).

For the separation of individual compounds, thin-layer electrophoresis-chromatography was used (19). Identical amounts of radioactivity were applied to thin-layer plates. Compounds were visualized by autoradiography and tentatively identified by co-chromatography. Sugars were co-chromatographed on silica (Stahl G type) gel plates in two dimensions (13). Amino and organic acids (15) were located with a ninhydrin spray (1 mg/ml) and an acid indicator (2, 7-dichlorofluorescein), respectively. Visualized and identified individual components were removed and counted by liquid scintillation. Quantitative data are expressed as percentage of total radioactive regions on each thin-layer plate.

Urea permeability.—Urea influx was measured using freshly excised hypocotyl cross-sections from healthy or virus-infected plants. Sections from several plants were pooled and preincubated in water for 30 min, blotted, and accurately weighed. Lots of 20 sections (150-200 mg, fresh wt.) were incubated in 1 ml 25 mM ^{14}C-urea (1 μCi/ml) at 25 C for appropriate times. Samples were shaken at 250 strokes/min. After incubation, the sections were washed for 15 min with water (five changes, 5 ml each rinse). The washed sections were extracted with 1 ml 95% ethanol overnight at −20 C, and the original extract and two 1-ml ethanol rinses for each sample combined in scintillation vials.

Urea efflux was measured as previously reported with some modification (9). Twenty hypocotyl cross-sections were incubated in 2 ml 50 mM ^{14}C-urea (20 μCi/ml) in cycloheximide (1 μg/ml) for 10 h at 25 C and washed 12 min (approx. 10 half-times for urea efflux from the apparent free space) in 20 mM urea (nonradioactive). After being washed, sections were resuspended in 2 ml of 20 mM urea (approx. isomolar with cell concn). Samples (10 μl) were withdrawn from bathing solutions at appropriate incubation times for radioactivity measurements. Two replicates of sections from healthy or SMV-infected plants were incubated in each experiment.

After efflux for 7 to 8 h, vials containing sections were incubated at 80 C for 5 min, destroying the resistance of cell membranes and allowing diffusion equilibrium between tissues and bathing solutions. Graphic

TABLE 1. Soil microbial populations around healthy and squash mosaic virus-infected squash hypocotyls

	Hypocotyls					
	0-3 mm		10-13 mm		Control[c]	
Microbes	H[a]	D	H	D	0-3 mm	10-13 mm
General bacteria	310.0[b]	960.0	71.30	67.1	62.0	67.00
Fluorescent pseudomonads	9.3	41.2	0.23	0.3	0.2	0.23
Actinomycetes	42.0	97.1	6.90	6.5	6.3	6.10

[a]Healthy (H) and squash mosaic virus-infected (D) plants.
[b]Microbes × 10^5/g soil.
[c]Soil population around glass rods.

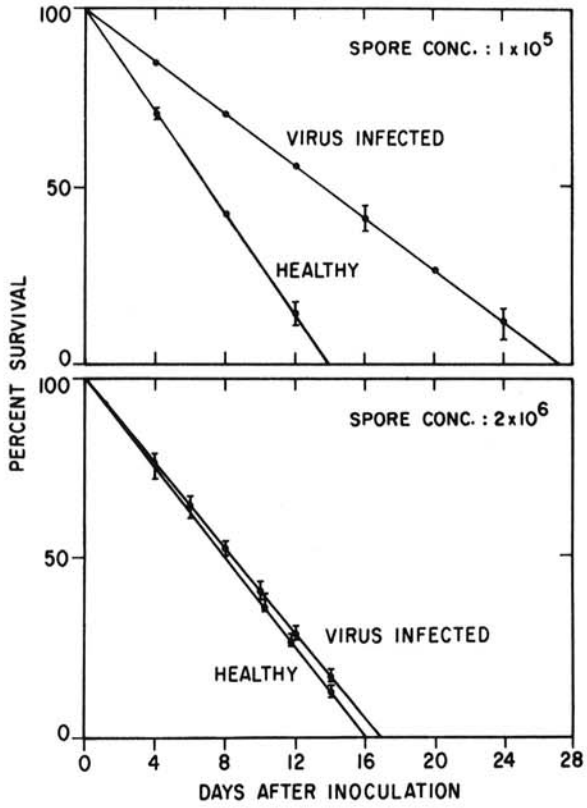

Fig. 1. Relationship between *Fusarium solani* f. sp. *cucurbitae* macroconidial concns and the degree of protection induced by squash mosaic virus. Data represent the average of 10 trials with 10 plants per trial. Virus-free plants served as the control. The vertical bars represent standard deviation.

presentation of efflux (S/S_e) is based on the levels (cpm/10 μl) of ^{14}C-urea in the bathing solution at each sampling time (S) and those at diffusion equilibrium (S_e).

Graphical analyses were made using the "peeling off" procedure (16). As shown in Fig. 4, straight-line portions of the graph observed at the late sampling periods (360-450 min) were projected backward (K2). Solute fractions at specific time values on the original curve (solid) were subtracted from solute fractions on the projected straight line (K2). Replotting the difference values on the log scale as a function of time yielded straight lines (K1). From the slopes of the original projected straight line (K2) and those derived by plotting the difference values (K1), rate constants (k) and half-times ($t_{1/2}$) for urea diffusion were calculated.

Uptake of ^{14}C-metabolites and analogues.—Procedures for measuring uptake of 3-*o*-methylglucose (MeG), a nonmetabolized glucose analogue, were described previously (8). In the present studies sections were incubated in 1 ml ^{14}C-MeG (2×10^6 cpm/μmole) in cycloheximide (1 μg/ml) for 1 h at 25 C. Procedures for washing and measuring radioactivity were identical to those used for urea uptake.

Techniques for measuring rates of uptake of 2-aminoisobutyric acid (AIB), an amino acid analogue which is not metabolized by plant tissue, were identical to those used for MeG. Specific activity of AIB was 2.6×10^6 cpm/μmole.

Uptake of other ^{14}C-amino acids (L-alanine, L-glutamate, and L-leucine), ^{14}C-organic acids (L-glycolate and L-malate), and ^{14}C-glucose was measured with the same techniques used for MeG. Normally 5 mM solutions (2 μCi/ml) were used.

Radioactivity measurements.—Radioactivity was measured by liquid scintillation in a dioxane (Aquascint II; International Chemical and Nuclear Corp., Cleveland, Ohio) or toluene (9) based scintillate (10 ml). Counting efficiency was about 60%. Radioactive compounds were purchased from International Chemical and Nuclear Corp., Cleveland, Ohio.

RESULTS.—*Microbial population.*—The squash hypocotyl has a distinct influence on microbial populations in soils 0 to 3 mm from the hypocotyl surface (Table 1). Differences in populations between soils adjacent to hypocotyls (0-3 mm) and glass rods (controls) were significant in over 30 separate experiments performed during the course of these studies. Moreover, bacteria and actinomycetes were significantly increased in the laimosphere of virus-infected plants compared with healthy plants. The microbe population expressed as laimosphere/soil ratio (L/S) [analogous to the rhizosphere/soil ratio (R/S)] was 4, 38, 6 for healthy and 14, 170, 15 for virus-infected plants, respectively, for general bacteria, fluorescent pseudomonads, and actinomycetes. Microbe populations in different samples 0 to 3 mm from hypocotyls were similar, but consistently different between healthy and diseased plants in 10 separate experiments. However, microbe populations

TABLE 2. Germination of chlamydospores of *Fusarium solani* f. sp. *cucurbitae* in water droplets on the surface of healthy and virus-infected squash hypocotyls[a]

Treatment	Experiment no. 1	2	3	4	5	$\bar{x} \pm m$
	Number of chlamydospores germinated after 12 h					
Control[b]	0	0	1	0	0	...
Healthy	62	64	61	60	61	61.6 ± 0.67
Virus-infected	64	61	63	64	62	62.8 ± 0.57

[a]Four droplets, each containing four to five chlamydospores, were placed on the hypocotyl surface. Each trial contained four healthy and four infected plants.

[b]Germination in water on glass rod.

periodically estimated in soils 3 to 10 mm from hypocotyls fluctuated from control levels to levels between controls and those in samples 0 to 3 mm from hypocotyls.

Protection in the pre-infection phase.—SMV-induced protection against stem rot caused by *F. solani* f. sp. *cucurbitae* depended upon the amount of inoculum added to the soil. When spore concn of inoculum was 2×10^6/ml, protection afforded by virus infection could not be observed. At a lower inoculum density (1×10^5), complete loss of virus-infected plants took an average of 27 days compared with 16 days for virus-free plants (Fig. 1).

Chlamydospore germination and penetration.—Microscopic examination revealed that chlamydospore germination of *F. solani* f. sp. *cucurbitae* was identical on SMV-free and SMV-infected plants (Table 2). When water droplets containing macroconidia on hypocotyl surfaces were allowed to completely dry during 4 to 6 h, the conversion of macroconidia and mycelial fragments to chlamydospores was rapid, but was neither increased nor decreased by virus infection. Little is known about host penetration by the fungus. Observations suggest that penetration often occurs through stomata. Mycelial cushion or appressoria formation by the fungus was not observed. Neither the number of initial lesions nor the number of dead cells associated with each lesion (recognized under the microscope as dark rectangular regions on hypocotyl surfaces) were changed by virus infection.

Exudation.—Fluctuations were observed in hypocotyl exudation patterns. The overall ratio of ^{14}C-labeled exudates between healthy and infected plants was 1:1.4. The amounts of ^{14}C-labeled water soluble compounds in exudates increased progressively in hypocotyl regions between the cotyledons and the root (Fig. 2). The highest amount of exudation was observed at the transition zone between root and hypocotyl. Chromatographic analysis of ^{14}C-labeled soluble compounds of hypocotyl exudates showed that there were little differences in the proportions of carbohydrates and amino acids given off by infected and healthy plants (Table 3). Concentrations of insoluble products in healthy hypocotyl exudates, probably lipids, unknown compounds and malate were slightly higher in healthy than in infected plant exudates. The qualitative composition of ^{14}C-metabolites in hypocotyl exudates was similar from healthy and diseased plants.

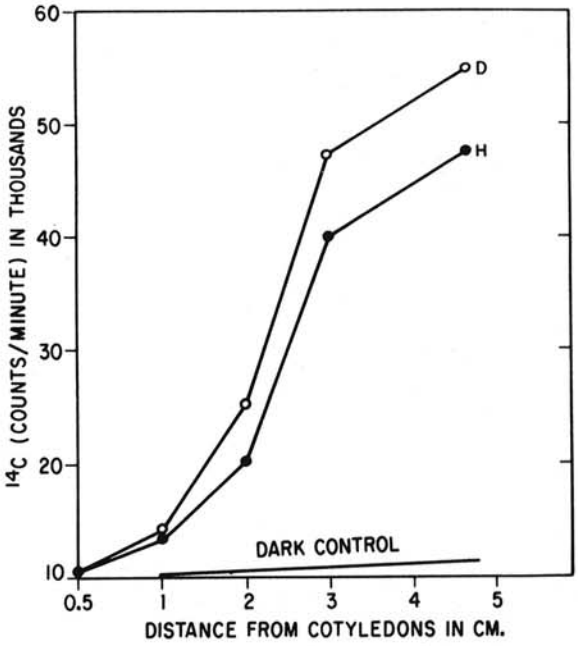

Fig. 2. Exudation of ^{14}C-assimilates from squash hypocotyls as a function of distance from cotyledons.

Large differences in proportions of metabolites in cellular extracts and exudates were noted. In both healthy and diseased plants amino acids made up a much larger proportion of ^{14}C-metabolites in exudates (40-50%) than in tissue extracts (2-5%), whereas glucose and fructose

TABLE 3. Distribution of ^{14}C-assimilates in hypocotyl exudates and extracts of healthy and virus-infected squash plants[a]

	Exudates		Extracts[b]	
Products	Healthy	Diseased	Healthy	Diseased
Alanine	18.0	21.0	0.5	1.2
Aspartate	16.3	9.8	0.3	1.0
Glutamate	9.1	5.8	0.9	2.9
Proline	3.1	1.6	0.2	0.4
Glycine and serine	0.9	0.5	0.1	0.2
Sucrose	10.0	8.7	7.1	4.8
Fructose and glucose	16.1	15.9	73.7	60.2
Malate	3.1	7.4	3.4	4.4
Glycerate	...	...	0.6	0.3
Phosphoenol pyruvate	...	...	1.5	3.5
Succinate	...	...	0.5	1.5
Sugar phosphate	...	...	0.2	1.1
Unknown organic acid	10.1	8.3	3.6	7.1
Unknown	0.3	0.8	2.9	3.0
Insoluble products	13.0	20.1	4.1	8.4

[a]Data is expressed as percentage of total ^{14}C activity.
[b]From 7-10% of the total radioactivity was found in other compounds not detectable in exudates.

composed a much higher proportion in tissue extracts (60-70%) than in exudates (16%). Sucrose composed a slightly higher proportion in exudates than in extracts.

Cell membrane permeability.—There were no consistent differences in the rates of diffusion of urea into (influx) or out of (efflux) hypocotyl sections from healthy and virus-infected plants (Fig. 3, 4). No differences in efflux of AIB or MeG were noted from sections from healthy or diseased plants preloaded with these compounds.

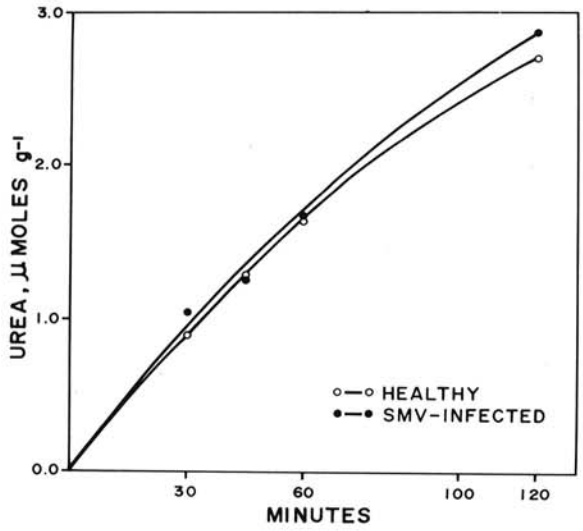

Fig. 3. Uptake of ^{14}C-urea by sections of subterranean portions of hypocotyls from healthy and virus-infected squash plants.

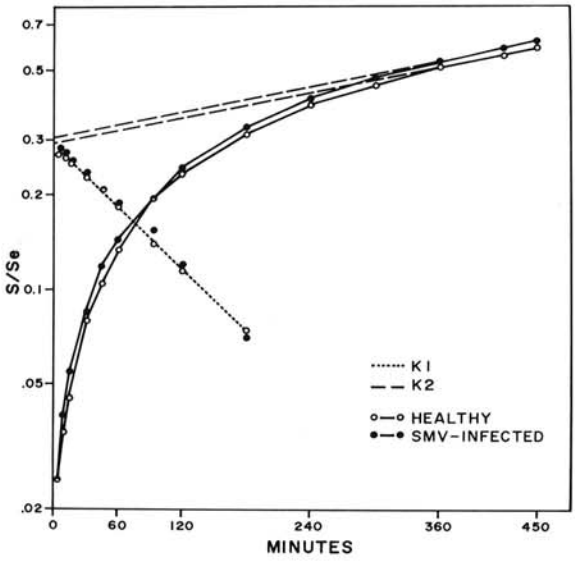

Fig. 4. Diffusion of preabsorbed ^{14}C-urea from healthy and virus-infected squash hypocotyl sections. The first exponential functions (K1) of the original curves were obtained by plotting differences between the original curves and backward projections (heavy dashed line) of the second exponential functions (K2).

When efflux of urea from hypocotyl tissues was plotted semilogarithmically, two major exponential functions were identified (Fig. 4). These functions apparently represent diffusion in series from two cellular compartments, the cytoplasm and vacuoles (11). Therefore, kinetic characteristics of efflux should reflect the permeability properties of membranes surrounding the cytoplasm and vacuoles, the plasmalemma and tonoplast, respectively. Squash mosaic does not affect the rate constants of diffusion across either of the limiting membranes (Fig. 4 and Table 4).

As shown in Table 4, the source of hypocotyl sections did not influence results. Rate constants of urea efflux across membranes in cells in subterranean (white) portions of hypocotyls from healthy or diseased plants were of the same magnitude as those in portions from above ground (green). Half-times ($t_{1/2}$) for urea diffusion from the cytoplasm ranged between 1 and 1.5 h, while $t_{1/2}$'s for diffusion from vacuoles were between 7 to 11 h (mean = 9.5 h).

Membrane transport systems.—Virus infection had no influence on the rate of uptake of MeG or AIB by hypocotyl sections over a range of solute concns (Fig. 5, 6). Moreover, no differences were noted in the capacity of healthy and diseased tissues to take up ^{14}C-labeled glucose, alanine, glutamate, leucine, glycolate, and malate.

DISCUSSION.—Virus-induced protection to Fusarium stem rot is not expressed during the hypocotyl penetration phase of pathogenesis. SMV-infection had no effect on the germination of *Fusarium* macroconidia or chlamydospores, fungal penetration, or initial lesion development when spores were placed on hypocotyl epidermal surfaces.

No evidence of enhanced postpenetration resistance was observed: lesion expansion rates and lesion staining characteristics in *Fusarium*-infected plants were identical in virus-free and SMV-infected seedlings. Minor changes in the proportions of cellular metabolites in hypocotyls were caused by SMV infection but the activities of membrane transport systems were identical. The lack of differences in disease development after penetration supports observations that host metabolic systems in the hypocotyls are not disturbed by SMV infection.

Diaz et al. (3) suggested that protection against Fusarium stem rot of squash was expressed during the prepenetration phase after noting that chlamydospore germination was reduced in soil surrounding the hypocotyl of virus-infected plants. In our work, chlamydospore germination in water droplets on the hypocotyl epidermis was not influenced by virus infection. These observations support the notion that protection is expressed during the activities of the pathogen in the laimosphere. The degree of protection afforded by virus infection is a function of inoculum level: in this study and that of Diaz et al. (3), protection evident at lower inoculum levels was nullified by higher inoculum levels. Thus, if the inoculum potential of *F. solani* is reduced below a critical threshold in the laimosphere as a result of virus infection, protection to stem rot is conferred.

TABLE 4. Rate constants for urea efflux from healthy and diseased squash hypocotyls

Material	Rate constants	Hypocotyl position: Subterranean (white)		Hypocotyl position: Above soil surface (green)	mean $t_{1/2}$[a]
		$10^{-3}min^{-1}$			min
Healthy	k_1	11.2	7.6	12.8	66
	k_2	1.2	1.6	1.0	530
Diseased	k_1	9.4	7.4	12.4	71
	k_2	1.1	1.5	1.1	580

[a]Half-times ($t_{1/2}$) were calculated from the mean of the rate constants from the three experiments.

The increase in exudation induced by virus infection does not directly reduce inoculum potential of the stem rot pathogen. Chlamydospore germination and pathogen infectivity are constant on hypocotyl surfaces of virus-free and virus-infected plants in the absence of soil. Exudation apparently indirectly influences inoculum potential via its influence on the laimosphere microflora. Microflora levels increase several fold in the laimosphere of squash hypocotyls as a result of virus infection which could exert a competitive influence on the stem rot pathogen during the initial phase of pathogenesis.

Exudation increases were directly related to increases in the cell permeability in roots of virus-infected peas (2). Although exudation rates increase in the squash system, there is no evidence that cell permeability or membrane

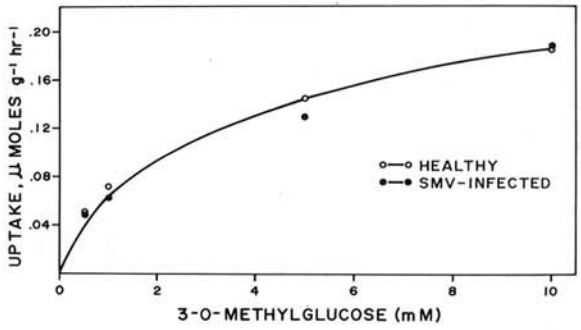

Fig. 5. Uptake of 3-*o*-methylglucose (MeG) by sections of hypocotyls from healthy and virus-infected plants as a function of external MeG concn.

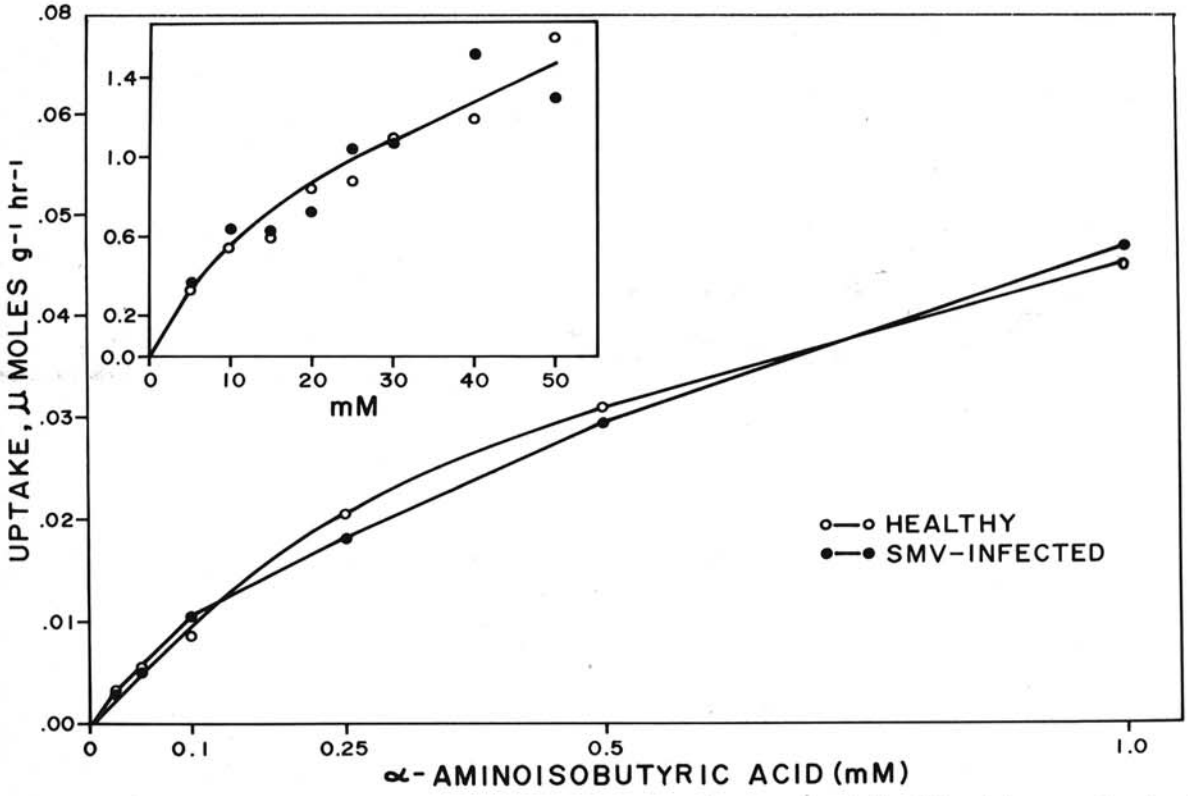

Fig. 6. Uptake of aminoisobutyric acid (AIB) by sections of hypocotyls from healthy and virus-infected plants as a function of external AIB concn.

transport is altered in hypocotyls after virus infection. It seems more likely that quantitative changes in hypocotyl exudation are caused by the substantial influence of virus infection on the physiology of squash leaves (13). Preliminary studies show that translocation of ^{14}C-assimilates to hypocotyls is enhanced by virus infection (A. C. Magyarosy, *unpublished*). Increased exudation after virus infection therefore may reflect the presence of increased levels of soluble metabolites in hypocotyl tissues.

The meaning of differences in the proportions of ^{14}C-labeled amino acids and sugars in hypocotyl exudates and extracts is not clear. Interpretation of these results is confused because the composition of exudates represents a composite profile of ^{14}C-assimilates collected over 36 h whereas tissue extracts were made 36 h after exposure and represent the relative composition of ^{14}C-assimilates in tissues at that time.

The subterranean stem is a principal infection court for numerous soil-borne pathogens. Yet little work has been done in plant pathology on soil microbes around stems and, in turn, on their influence on the behavior of stem pathogens. We have found that subterranean squash hypocotyls influence the populations of the microflora in the surrounding soil, a region we have designated the laimosphere (12). The laimosphere imposes a strong influence on fungal disease development in the SMV-*Fusarium* disease complex and we feel that hypocotyl exudation is responsible for this effect. However, much more research is required before clear conclusions can be drawn on the function of the laimosphere and, particularly, its role in plant protection.

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