Effects of Infection by Peanut Mottle Virus on Nodule Function

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ABSTRACT

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The effects of infection by peanut mottle virus on nodulation and nodule function of cultivar Early Prolific peanuts, inoculated with either effective or ineffective strains of *Rhizobium* and grown under controlled conditions, were assessed at the early blooming, late blooming, pegging, and pod-filling stages. Virus-infected plants showed differences in magnitude of growth reduction and nodulation pattern, depending on whether they harbored the effective or ineffective rhizobial strain. In plants infected with effective rhizobia, the commencing of nitrogenase activity (estimated by measuring C_2H_2 reduction) was delayed, and the activity measured on a per plant basis was reduced, but the maximum nodular specific activity per milligram of nodule tissue was not significantly lower than that of healthy plants. There was no correlation between leghemoglobin content and the nitrogenase-specific activity of nodules from plants infected with peanut mottle virus, but a positive correlation was observed in the healthy controls. Assays indicated the presence of infective peanut mottle virus in tissues of both effective and ineffective nodules.

Additional key words: *Arachis hypogaea*, bacteria-virus interaction.

Peanuts (*Arachis hypogaea* L.) are an economically important pulse legume, particularly in developing countries, where production has increased steadily in recent years (3). Peanut mottle virus (PMV), a potyvirus, is worldwide in distribution, and can cause considerable yield losses (4). Mild strains of the virus are more widespread than severe ones in the U.S., where five serologically indistinguishable strains have been differentiated by symptom expression in peanuts (22,28).

Biological nitrogen fixation is regarded as an important process in leguminous plants (31). Physiological and environmental stress factors affecting nodulation and nodule function have been listed by several reviewers (7,18,23,27). However, the effects of virus infection were included in only one of the lists (11).

Various reports have shown that legume nodule structure and function are affected by virus infection and much of the available information has been summarized recently (29). Most of the reports involved host plants on which exogenous nodules developed after the formation of infection threads by entering rhizobia. Peanut nodules develop after passive invasion by rhizobia at sites of lateral root emergence (6) and thus have been classified as endogenous (1). It has been suggested that the simplicity of the rhizobial infection process decreases the degree of specificity between the symbionts (7), and the results of some studies have indicated that peanuts nodulate readily with rhizobial strains in various cross-inoculation groups (2,25).

This investigation was undertaken to examine the effects of infection by PMV on peanut nodulation, growth, nitrogenase activity, and leghemoglobin accumulation. Effective (i.e. nitrogen fixing) and ineffective strains of *Rhizobium* were used; the influence of rhizobial strains on symptom expression has been reported elsewhere (36).

MATERIALS AND METHODS

Culture of plants, *Rhizobium*, and virus. Washed, rhizobia-free seeds of cultivar Early Prolific peanut were pregerminated in autoclaved, washed sand for 4 days. Germinating seeds with 4- to 5-mm-long hypocotyls were then harvested and inoculated with rhizobia.

Two strains of a slow-growing *Rhizobium* sp., representing effective and ineffective microsymbionts, were used for inoculation. The effective strain, designated 8A54, was selected from four others (generously provided by J. C. Burton, Nitragin Co., Inc., Milwaukee, WI) because it showed the highest nitrogenase-specific activity (estimated by measuring C_2H_2 reduction) and stimulated maximum leghemoglobin production in the nodules. The ineffective strain, ATCC 10317, was purchased from the American Type Culture Collection, Rockville, MD. Both strains were isolated from single colonies cultured on yeast-extract mannitol agar (33,34) and used as seed inoculants as described previously (36).

Two seeds inoculated with rhizobia were sown in each of 96 clay pots (15-cm diameter) containing an autoclaved sand:loam soil mixture (1:1, v/v), pH 6.2, with a total nitrogen content of about 0.02%. Plants were kept under controlled conditions: 10,000 lux light intensity for 12 hr/day, 28/25 C day/night temperature, and 75–85% relative humidity. One hundred milliliters of a quarter-strength, nitrogen-free Long Ashton solution (8) was added to each pot twice, 2 wk apart. Sterilized distilled water was used to irrigate the plants for the first 2 wk after planting, after which tap water was used.

A mild strain of PMV, designated as PMV 348TC2 (kindly supplied by S. A. Tolin, Virginia Polytechnic Institute and State University, Blacksburg) was used as a source of virus inoculum. Reisolation from a single lesion, culture maintenance, and inoculation of peanut plants were as described (36).

Assessment of plant growth. Each treatment, i.e. PMV-infected or healthy control, consisted of 96 plants, inoculated with effective or ineffective *Rhizobium* depending on the experiment. Treatments were randomized into four replicates, each consisting of 24 plants (12 pots). Four samplings were made on each treatment, three pots (six plants) being randomly chosen from each replicate. Growth parameters of plants with effective microsymbionts were determined at the early blooming, late blooming, pegging, and pod-filling stages, which occurred at 37, 51, 72, and 92 days, respectively, after seeding. In plants with ineffective microsymbionts, the first three observations were made at 28, 42, and 61 days because root system development and flowering occurred earlier; the last observation was made at 92 days.

At each growth stage, plants were randomly collected, removed from pots, and washed free of soil with running water, which was

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passed through a sieve to collect root fragments and detached nodules. Roots were blotted dry and detached from shoots just below the cotyledons. Fresh weights of roots, shoots, and pods were recorded separately. Data were obtained for each of four replicates of six pooled plants in each treatment; one-tailed Student's *t*-tests were used to examine differences between treatment means.

Nodules were harvested within 24 hr of plant collection and after roots had been processed for nitrogenase activity (C_2H_2) assay. They were detached from roots of six pooled plants with a razor and kept in a miniature moist chamber in an ice bath. Nodule yield was assessed as fresh weight or number per six plants from which the average individual nodule weight in milligrams fresh weight per nodule was determined.

Assay of nitrogenase activity. The nitrogenase activity of effective nodules on excised roots was determined by the acetylene (C_2H_2) reduction test (13). Ten milliliters of 101.28 kPa (1 atm.) acetylene were injected into a 250-ml flask containing the roots and nodules, which was incubated for 45 min at room temperature, with shaking at 5 min intervals. A sample of 0.2 ml of gas was withdrawn and injected into a Hewlett-Packard gas chromatograph with a 2-m × 3-mm Durapak column operated at 50 C and with helium as a gas carrier. The amount of ethylene (C_2H_4) produced by each sample was calibrated by comparing measured peak heights to a standard concentration curve (5). Each assay included a sample of 0.2 ml standard 0.01% C_2H_4 as a fluctuation check. Plant nitrogenase activity was expressed as nanomoles of ethylene produced per plant per minute, and nitrogenase-specific activity was expressed as nanomoles of ethylene produced per milligram of nodule fresh weight per minute. Data are given as means of activities from four replicates, each consisting of six nodulated roots.

Determination of leghemoglobin. The leghemoglobin content of effective nodules was determined by a fluorometric assay (17). Approximately 25% of the pooled nodules from each replicate were homogenized at moderate speed with a Tissue-mizer (Tekmar Co., Cincinnati, OH) in 30 volumes (v/w) of extracting medium; further sample preparation was as described by La Rue and Child (17). Fluorescence of samples was measured with a Turner fluorometer with excitation and emission wavelengths of 405 and 595 nm, respectively. A similarly-treated mixture of extracting medium and oxalic acid was used as a blank, and a root homogenate was included as a control. Readings were converted to leghemoglobin content by comparison with a standard concentration curve for beef blood hemoglobin (Sigma Chemical Co., St. Louis, MO). Data are given as micrograms of leghemoglobin per milligram fresh weight of nodule averaged from four replicates of six pooled plants.

Estimation of viable rhizobial population. Approximately one quarter of the nodules from each replicate were surface disinfested, aseptically transferred to 250 volumes (v/w) of autoclaved 1% mannitol, and comminuted at low speed with a Tissue-mizer. Three 10-fold serial dilutions of this stock were made with 1% mannitol. Subsequently, 0.1 ml of the stock suspension or of the 10^{-3} dilution was spread on the surface of a hardened congo-red, yeast-extract mannitol agar plate. Three plates from each sample were inoculated and incubated at 27 C for 7–8 days, after which visible colonies were counted. Results represent colony number, in thousands, per milligram of nodular tissue, averaged from four replicates.

Virus infectivity assay. The relative virus content of leaves, roots, and nodules, was estimated by infectivity assays. Leaf disks (3 mm in diameter) were taken from the central area, adjacent to the midrib, from either side of the second compound leaf above the PMV-inoculated leaf. Root tissues were taken from the area about 5 mm above the nodules. Nodules were those used for nodule yield assessment. One hundred milligrams of each tissue were withdrawn from the pooled sample and homogenized in 0.5 ml of 0.05 M potassium phosphate buffer (pH 7.5, containing 0.1% sodium sulfite) in a miniature mortar and pestle consisting of a small round-bottom glass tube and a round glass rod having a slightly smaller diameter. The resulting extract from each sample was further diluted with 0.5 ml of buffer and clarified by centrifugation at 9,750 *g* for 10 min. The supernatant was mixed with 10 mg of Celite, and 20 μl was applied to each half of primary leaves of 10-day old Topcrop beans (*Phaseolus vulgaris* L.) and spread on the surface with a glass roller. Inoculum residue was washed from the leaves with distilled water, and the plants were returned to the growth bench under controlled conditions of 10,000 lux light, 12 hr day, 28/25 C day/night temperature, and 75–85% relative humidity. Necrotic local lesions were counted 3–4 days after inoculation. Ten half-leaves were used to assay each inoculum preparation and the arrangement of inoculation was in randomized complete block fashion as described by Kleczkowski (15). The fourth half-leaf of each plant was inoculated with buffer inoculum as a control.

RESULTS

General characteristics of rhizobia-inoculated, healthy peanut plants. Plants inoculated with the ineffective *Rhizobium* ATCC 10317 grew rather poorly. Their lowest leaves were paler than those of plants inoculated with the effective *Rhizobium* 8A54, stems were less branched, and the flowering stage was initiated earlier (about 28 days after planting). Although numerous flowers were produced on plants harboring ineffective microsymbionts, only a few of them developed to the pegging stage.

Numerous small white nodules developed on the entire root system of plants with ineffective *Rhizobium*. No leghemoglobin was detected in them, and only negligible nitrogenase activity was detected at any growth stage. In contrast, plants inoculated with effective *Rhizobium* 8A54 grew vigorously and bore fewer (but larger) nodules which were usually pink. Most of these nodules were formed on the main and secondary roots, with relatively few on the tertiary roots (Fig. 1).

Effects of infection by PMV on rhizobia-inoculated peanuts. *Plant growth.* In plants inoculated with the effective *Rhizobium*, PMV did not reduce shoot growth (Fig. 2A). Root fresh weights were reduced at all stages, but the reduction at early blooming was not statistically significant. Pod fresh weight was also reduced (15.69 ± 1.99 g versus 21.11 ± 0.65 g). The total weight reduction (root plus shoot) reduction in PMV-infected plants at 92 days was about 14%.

In plants with the ineffective *Rhizobium*, infection by PMV reduced shoot growth at the last two stages of growth (Fig. 2B). Root fresh weight reductions occurred at all stages, but the reduction at 42 days was not statistically significant. The total weight reduction caused by infection by PMV was about 25% at 92 days.

Nodulation. The average weight of effective nodules on PMV-infected plants was less than that of healthy controls at 37, 51, and 92 days, but was not different at 72 days (Fig. 3A). Nodule fresh weight (milligrams per six plants [*unpublished*]) and number were

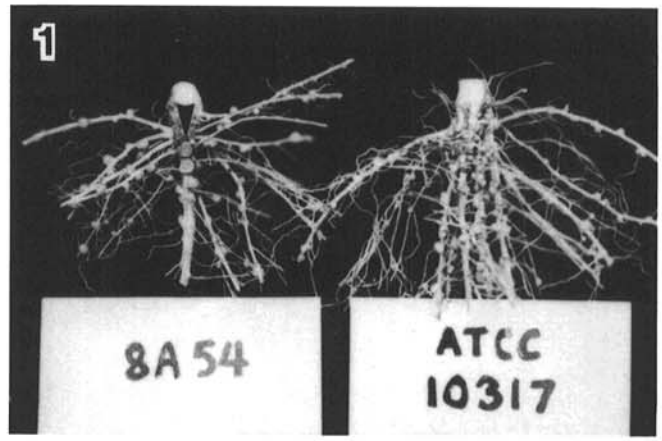

Fig. 1. Nodule distribution on healthy cultivar Early Prolific peanut roots inoculated with either the effective strain (8A54) or the ineffective strain (ATCC 10317) of *Rhizobium* and collected at late blooming. Fewer effective nodules (left) developed, but they were larger and more pigmented (arrow head).

also lower in PMV-infected plants when measured at 37 and 51 days but were not different at later times (Fig. 4A). The average weight of nodules (milligrams per nodule) and nodule fresh weight (milligrams per six plants) of healthy plants were maximum at 51 days. However, the maximum average weight of nodules on infected plants was reached at 72 days (1.80 ± 0.15 mg), when it was only slightly greater than at 51 days (1.75 ± 0.14 mg [Fig. 3A]).

PMV-infected plants with ineffective nodules showed an apparent increase in nodule average weight, compared to healthy controls at 28 days, but close observation revealed that these large "nodules" were actually clumps of many very small nodules. This clumping did not occur in healthy plants or at later stages in infected plants, and posed less of a problem during nodule collection at the later stages when relatively minor differences in nodule average weight between PMV-infected and healthy control plants were noted (Fig. 3B). Nodule fresh weight (milligrams per six plants) was significantly reduced at 42, 61, and 92 days (*unpublished*) in virus-infected plants. Numbers of nodules formed on infected plants (Fig. 4B) were significantly less than those on healthy plants at all times, and nodule numbers reached their maxima in infected and control plants at the same times. At 42 days, infection by PMV caused about 42 and 44% reduction in nodule fresh weight and number, respectively.

Nitrogenase activity (C_2H_2). The nitrogenase-specific activity

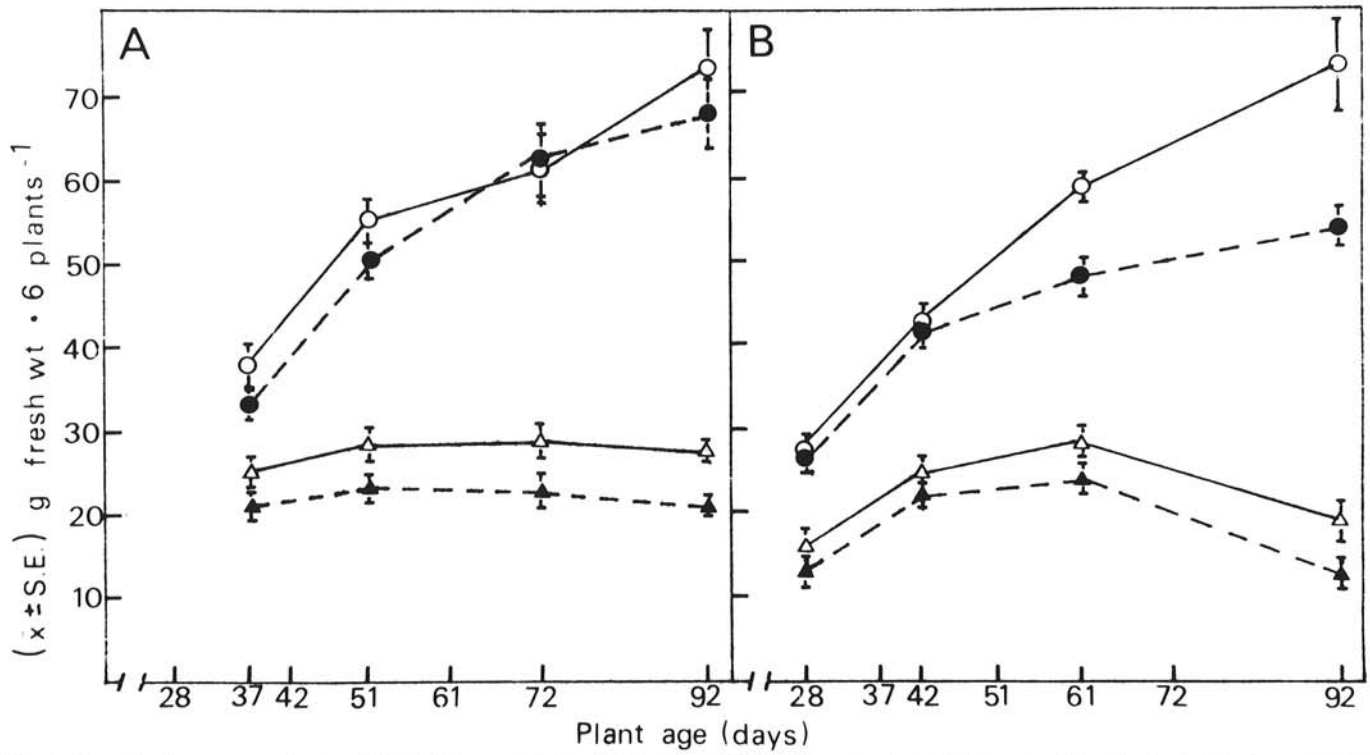

Fig. 2. Growth of peanut mottle virus-infected (------; solid symbols) and uninfected (—; empty symbols) roots (Δ) and shoots (o) of peanut plants inoculated with **A,** effective and **B,** ineffective rhizobial strains.

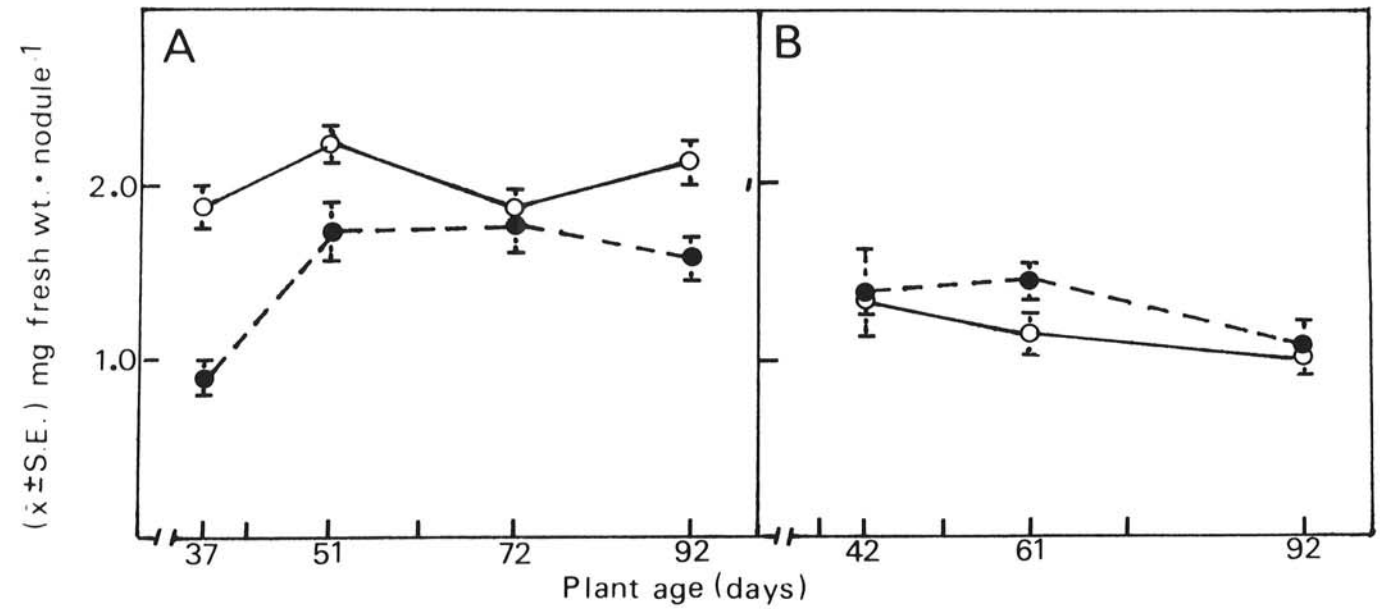

Fig. 3. Average individual nodule weights of peanut mottle virus-infected (•——•) and uninfected (o——o) peanut plants inoculated with **A,** effective and **B,** ineffective rhizobial strains.

(Fig. 5A) of nodules from effectively nodulated PMV-infected plants was lower than that of nodules from healthy plants at early blooming (37 days) and late blooming (51 days). Nitrogenase activity expressed on a per plant basis was significantly lower in PMV-infected plants during the first three growth stages but not at pod filling (Fig. 5B). Activity reached its peak at late blooming (51 days) in both treatments, and nitrogenase activity on a per plant basis was reduced by about 65% at this stage in infected plants.

Leghemoglobin content. The leghemoglobin content of effective nodules from PMV-infected plants was significantly lower than that of healthy controls at early blooming ($P < 0.05$) and late blooming ($P < 0.01$) but was less different at pegging and pod filling (Fig. 6). In healthy nodules, the leghemoglobin content reached its peak (0.84 ± 0.13 μg per milligram of nodule) at 51 days, then declined at 72 days and rose again at 92 days. Nodules from PMV-infected plants showed no such cycle but instead displayed a steadily increasing leghemoglobin content. At 51 days, the leghemoglobin content of infected plants was 63% lower than that of the healthy controls.

Nitrogenase activity and leghemoglobin content both increased between 37 and 51 days. However, plots of nitrogenase-specific activity against leghemoglobin content during the entire observation period (*unpublished*) indicated no correlation between the two in PMV-infected peanuts ($r = 0.169$), but a significant ($P < 0.05$) positive correlation ($r = 0.763$) in the control plants.

Nodular rhizobial population. Rhizobial population assessment of effective nodules could not be done at 37 days because nodules of PMV-infected plants were small and few could be collected. Infected and healthy plants were not different at 51 and 72 days (Table 1), but the population in nodules from healthy plants was smaller ($P < 0.05$) than that from infected plants at 92 days.

In the ineffective nodules, the rhizobial population in nodules from healthy plants was higher than that from PMV-infected plants ($P < 0.01$) during the first three growth stages, but was not different at 92 days (Table 1).

Virus infectivity assay. In plants with the effective *Rhizobium*, the virus infectivities in root and nodular tissues were comparable (Fig. 7A) and less than that of leaves ($P < 0.05$) at early blooming (37 days, i.e. 20 days after inoculation with the virus). Subsequently, the infectivity of root and leaf tissue dropped, while that of nodules increased before dropping after late blooming. No infectivity was detected in root tissue either at or after the pegging stage.

In plants with ineffective *Rhizobium*, the magnitude of virus infectivity was in the order: leaf tissue, nodular tissue, and root tissue ($P < 0.05$) at all stages (Fig. 7B). Leaf infectivity was about the same at early and late blooming stages, then declined. In nodular and root tissues, the virus infectivity declined sharply after early blooming. Only slight infectivity was recovered in nodular tissue, and none was detectable in root tissue either at or after late blooming.

DISCUSSION

The experiments reported here revealed various differences between PMV-infected and healthy plants, as well as between those harboring effective and ineffective *Rhizobium*. Infection by the virus did not significantly reduce shoot growth in effectively nodulated plants, but root and pod weights were reduced. Kuhn (16) reported similar results from work with three peanut cultivars inoculated with a mild strain of PMV. The reduction in root growth was not significant until after early blooming, when nitrogen fixation was increasing, which may indicate that infection by the virus exerts a greater effect on this process than on the use of stored reserves and combined soil nitrogen during the earlier stages of growth.

Virus-infected plants with ineffective *Rhizobium*, which showed significant decreases in both shoot and root growth at later stages of growth, also showed more severe foliar symptoms (36). The greater total weight reduction associated with virus infection in these plants suggests that in effectively nodulated plants, nitrogen fixation may have offset the effects of infection by PMV. Conversely, an inadequate supply of nitrogen may have aggravated the effects of infection by PMV in the plants with ineffective *Rhizobium*.

The average weight of individual effective nodules was reduced by approximately 50% at early blooming in PMV-infected plants. These nodules reached their maximum weight at 72 days, while those of healthy plants reached their maximum average individual

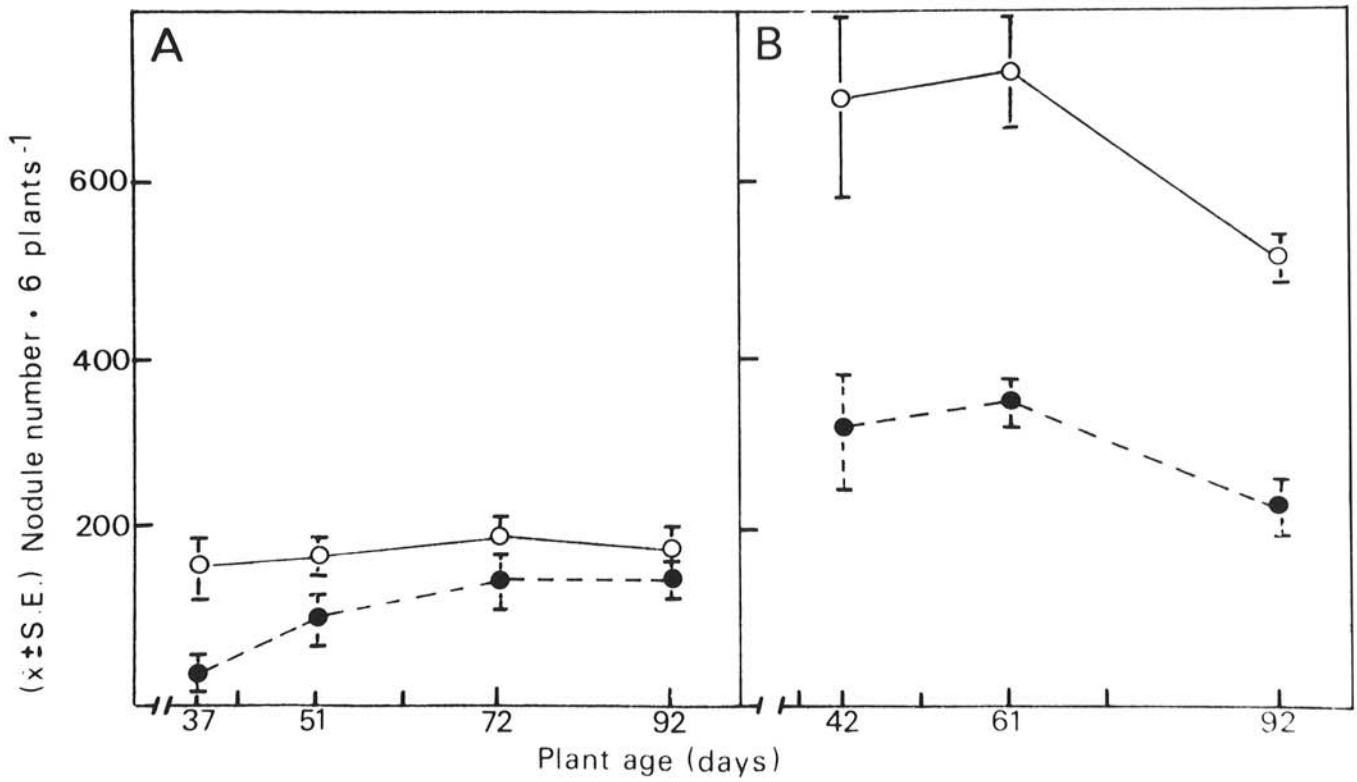

Fig. 4. Numbers of nodules developed on roots of peanut mottle virus-infected (●——●) and uninfected (o——o) peanut plants inoculated with **A,** effective and **B,** ineffective rhizobial strains.

weight at 51 days. Infection by PMV may have suppressed or delayed the development of effective nodules. Such a suppression has been reported in other virus infections (14,21).

Infection by PMV seemed to affect ineffective nodulation somewhat differently, as nodule size was not reduced, but nodule number was reduced considerably at all stages. This contrasts with the effect on effectively nodulated plants, in which nodule growth was apparently retarded, but the number of nodules present was not significantly reduced at later growth stages. These differences probably reflect differences in nodule distribution. One of the characteristics of ineffective peanut nodulation is the distribution of small nodules over the entire root system (2). This was observed in healthy control plants with ineffective nodules, but PMV-infected plants resembled effectively nodulated plants in that nodules were limited to the main and secondary roots. However, numerous abortive nodules could be detected on tertiary roots; possibly their further development had been suppressed by infection with PMV.

The profiles of nitrogenase (C_2H_2)-specific activity and plant nitrogenase activity at various growth stages of peanuts from both treatments are similar to those reported for Virginia Jumbo peanuts by Hardy et al (12). Activity was initiated at early blooming, reached its peak at late blooming, and declined at pegging. By pod filling (92 days), the activity was negligible.

The lower nitrogenase-specific activity of nodules from PMV-infected plants relative to that of healthy controls at early blooming may indicate a delay of nodule development rather than reduced nitrogenase efficiency as the nodules from infected plants were considerably smaller at this stage (Fig. 3A). Similarly the lower plant nitrogenase activity of infected plants observed at the first three growth stages may be attributable to their lower nodule weight and number. Reductions in nitrogenase activity associated with virus infection have also been reported for nodules of soybean (21), beans (20), and red clover (14) infected with certain viruses. The overall decrease in nitrogenase activity has generally been attributed to virus-caused impairments or reductions of photosynthetic activity, as this is a common aspect of plant virus infection (19). This does not preclude the possibility that plants may be able to nullify or offset the effect of virus infection upon nitrogenase activity by other means.

The fluorometric method for determination of leghemoglobin offered a sensitive assay, applicable for use with small nodules, as consistent readings were obtained with beef hemoglobin concentrations as low as 0.1 $\mu g \cdot ml^{-1}$. The effect of infection by PMV was interesting in that the positive correlation between leghemoglobin content and nitrogenase specific activity of nodules shown by healthy plants was lacking in the virus-infected plants. Thus, the synchronization of leghemoglobin production and nitrogenase activity, which generally is encountered in legume symbiosis (9), may have been disrupted by the viral infection. However, the lower leghemoglobin content of infected plants at late blooming was apparently sufficient for maintenance of nitrogenase activity, as the latter reached its highest value at this stage. Other investigators have also noted reductions of

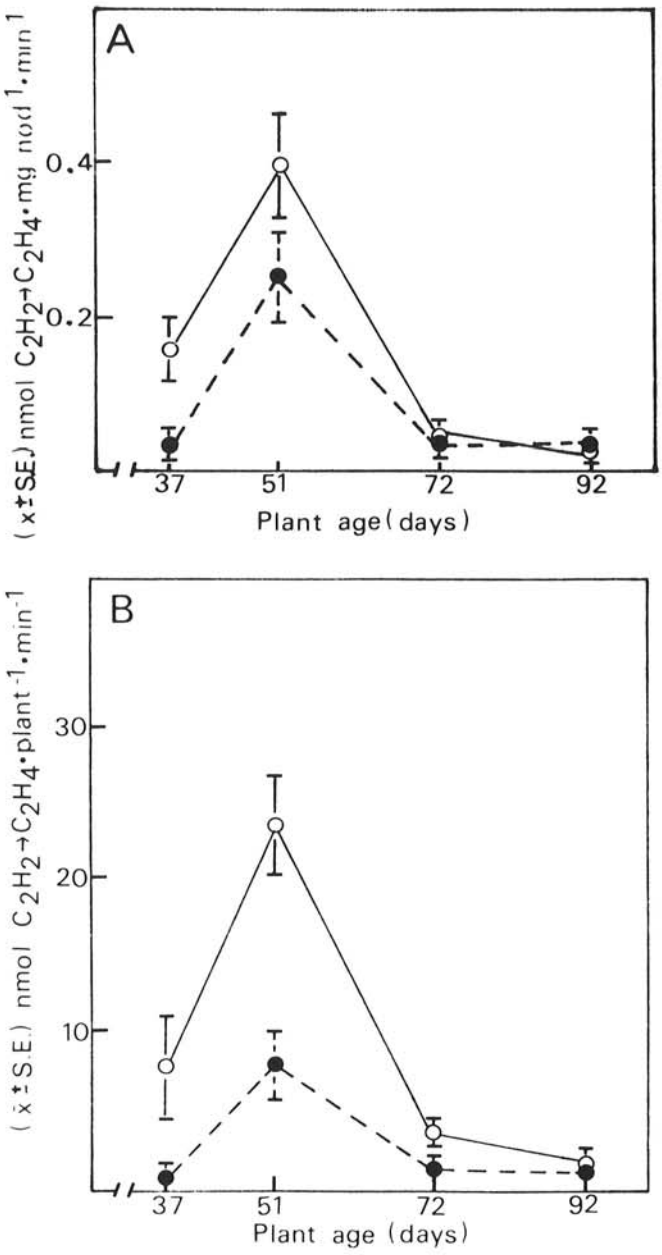

Fig. 5. Profiles of nitrogenase-specific activity of peanut mottle virus-infected (●——●) and uninfected (o——o) peanut plants inoculated with effective rhizobial strain 8A54. Expressed **A**, per milligram of nodule fresh weight and **B**, on a per plant basis.

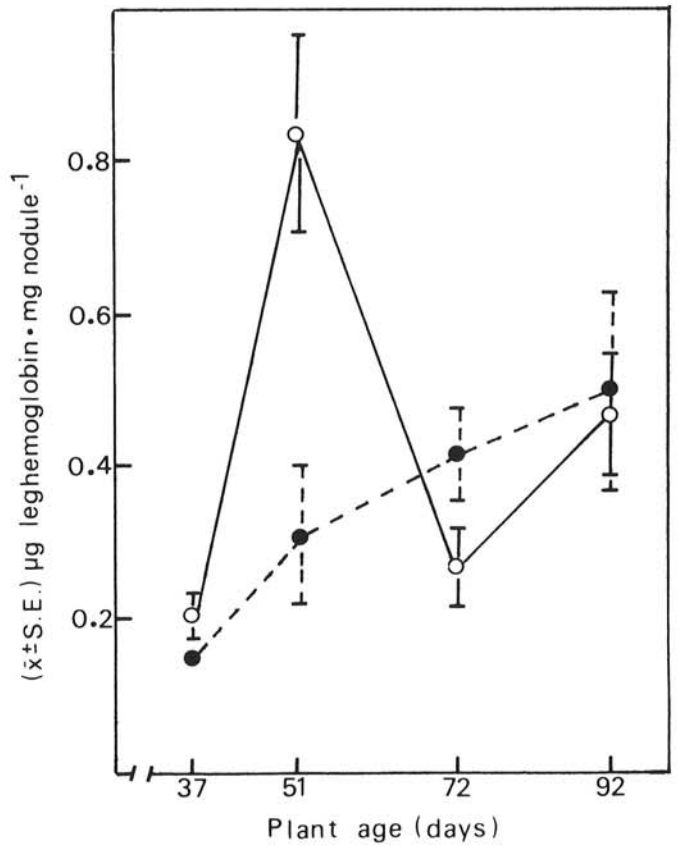

Fig. 6. Leghemoglobin content of nodules from roots of peanut mottle virus-infected (●——●) and uninfected (o——o) peanut plants inoculated with effective rhizobial strain 8A54.

leghemoglobin content in nodules of virus-infected red clover (14) and soybeans (30). On the other hand, Orellana et al (21) noted an increase in leghemoglobin content in nodules of tobacco ringspot virus infected soybeans, and a negative correlation between leghemoglobin content and nitrogenase activity in both healthy and infected soybeans. However, the colorimetric assay (35) used in their study does not differentiate between leghemoglobin and biliverdinlike pigments in aging nodules (17) which may lead to overestimates of leghemoglobin during senescence.

The numbers of viable rhizobia isolated from 1.0 mg of tissues from both ineffective and effective nodules are within the range reported in various peanut cultivars (26), although different isolation techniques were used, indicating that the latter did not affect the results. Virus infection consistently caused a greater reduction in the population of free-living rhizobia in ineffective nodules than in effective ones, which may indicate an insufficient supply of nutrients in the former. Low concentrations of bacteria other than the slow-growing rhizobia were also recovered from the diluted suspension prepared from nodules at the last growth stage (92 days). Extensive identifications were not undertaken, but colony appearance, cell morphology, and Gram stain reaction suggested that these "contaminants" could be *Bacillus* spp., a fast-growing gum-producing *Rhizobium* sp., and perhaps an *Azotobacter* sp. These groups have been encountered by various investigators (10,32), even from nodules collected from legumes grown under strictly aseptic conditions.

The profiles of PMV infectivity in leaf tissue resemble those reported earlier in various peanut cultivars and peas (22), in that infectivity was high during the early period after infection, and then declined considerably. Similar declines also took place in nodular and root tissues. The increased virus infectivity of nodular tissues of plants with effective *Rhizobium*, observed at late blooming, is interesting in that it coincided with maximum nitrogen fixation activity of the nodules. This suggests that virus can multiply in nodular tissues and may indicate that active nitrogen fixation supports viral multiplication.

TABLE 1. Effect of infection by peanut mottle virus (PMV) on rhizobial populations in peanut nodular tissues observed at different growth stages

	Colonies ($\times 10^3$) per milligram of nodular tissue at:			
	51 days	72 days		92 days
Effective nodules				
Control	507.75 ± 104.90	246.05 ± 36.60		109.79 ± 24.21
PMV-infected	345.83 ± 112.18	270.63 ± 35.67		494.16 ± 141.74
Computed *t*	0.982	0.481		2.747
Level of significance[a]	N.S.	N.S.		*
	28 days	42 days	51 days	92 days
Ineffective nodules				
Control	1,033.50 ± 60.56	340.00 ± 51.87	392.71 ± 26.40	386.77 ± 48.78
PMV-infected	486.62 ± 110.07	128.12 ± 30.88	129.78 ± 21.15	420.78 ± 31.44
Computed *t*	4.337	3.510	7.770	0.586
Level of significance[a]	***	**	***	N.S.

[a]One-tailed critical *t* values: *t* 0.05(6) = 1.943 (*); *t* 0.01(6) = 3.143 (**); and *t* 0.005(6) = 3.707 (***). N.S. = nonsignificant.

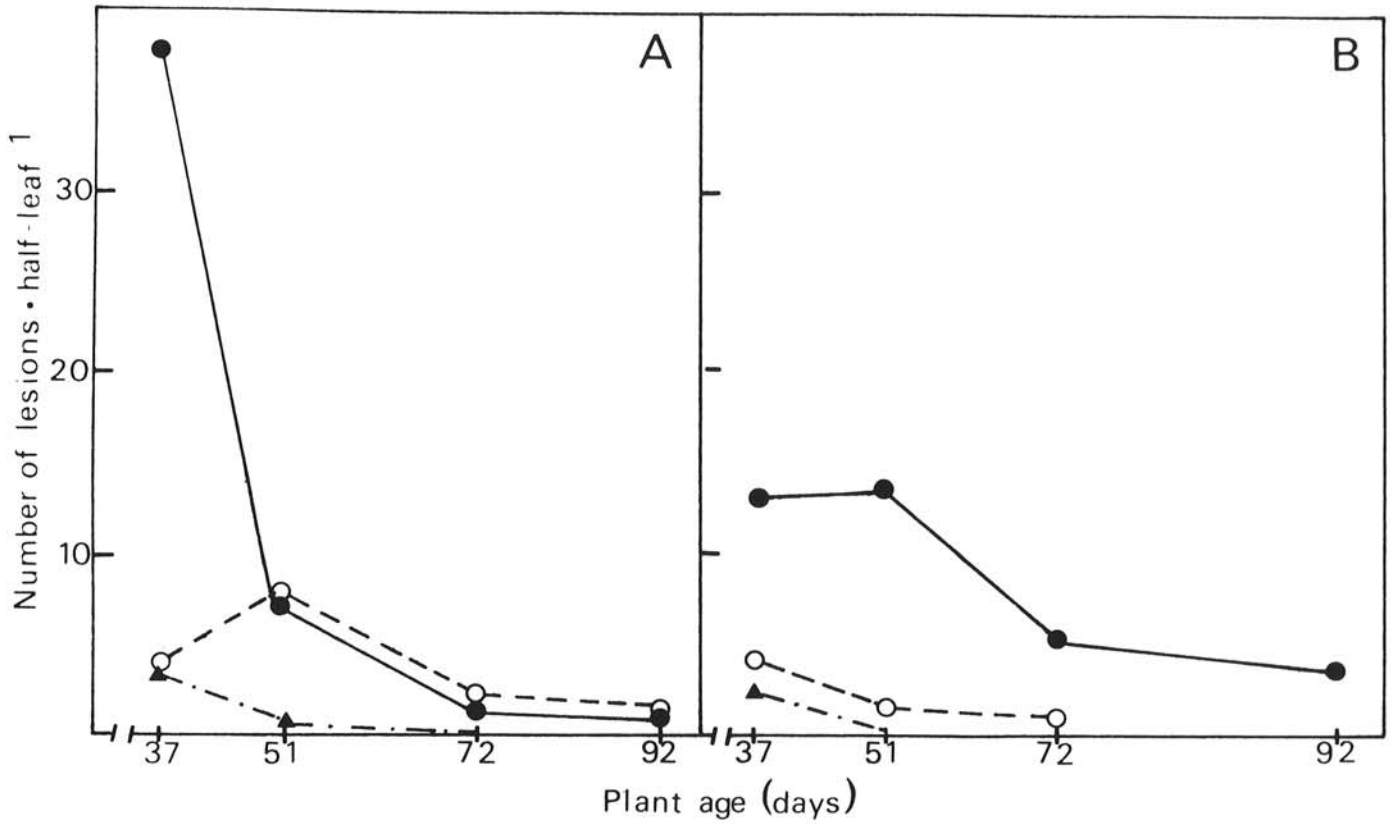

Fig. 7. Assays of peanut mottle virus infectivity in leaf (●——●), nodule (o——o), and root (▲-·-·-▲) tissues of peanut plants inoculated with **A**, effective and **B**, ineffective strains (8A54 and ATCC 10317, respectively) of rhizobia.

The suppression of nodule growth and development, reduction of nitrogenase activity on a per plant basis, and an alteration of the leghemoglobin production pattern all indicate that the infection by PMV affects symbiotic nitrogen fixation to a certain extent. Numerous factors, both biological and physical, are known to affect either nodulation (11) or plant reaction to virus infection (19). Thus, it is perhaps inappropriate to conclude that the effects of infection by PMV observed in this investigation are universal. However, if virus infection had completely impaired nitrogen fixation, the yield of plants infected with the effective *Rhizobium* should have been comparable to that of their counterparts with the ineffective *Rhizobium*. The smaller yield reduction of the former suggests that nitrogen fixation can partially offset the effect of infection by the virus. Similar findings have been reported from work with virus-infected *Dolichos lablab* inoculated with effective rhizobia (24). The immediate implication is that selection of peanut lines tolerant or resistant to infection by PMV could be complemented by searching for rhizobial strains that can assist in compensating for effects of virus infection.

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