Tobacco Ringspot Virus and Rhizobium Interactions in Soybean: Impairment of Leghemoglobin Accumulation and Nitrogen Fixation

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ABSTRACT

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Tobacco ringspot virus (TRSV)-infected and noninfected Harosoy soybeans grown in the greenhouse with *Rhizobium japonicum*-110 seed-inoculant were studied during prebloom, bloom and seed-set, bloom and early pod, pod-fill, and mature-pod stages. The effects of the disease relative to noninfected plants were the following: (i) the virus significantly reduced top, root, and nodule growth; (ii) nodulation and hence symbiotic activity were nearly or completely suppressed until the plants were about 40 days old and were in the bloom and early pod stage; (iii) after nodulation started, nodule fresh weights were reduced 85, 67, and 67% during the last three growth stages; (iv) leghemoglobin (LH) content decreased 3% during the bloom and early pod stage but increased 33 and 25% during the successive pod-fill and mature-pod stages, respectively; (v) N_2-fixation rates calculated in micromoles of C_2H_4 · gram-nodule^{-1} · hr^{-1} (on a fresh-weight basis) were increased 30, 99, and 57%, respectively, during the last three growth stages as the plants aged; however, N_2-fixation rates, calculated on plant basis for the last three growth stages, decreased 81, 41, and 23%; and (vi) TRSV-infected plants remained green 2-3 wk longer than the noninfected controls. Correlation coefficients between LH accumulation and N_2 fixation rate for TRSV-infected and noninfected plants were $r = -0.9900$ and -0.8905, respectively. These results demonstrate that soybean budblight disease severely delays nodulation and interferes with the efficiency of the N_2-fixation process. The delayed, although increased, specific activity of TRSV-infected nodules would be too late to promote yield.

Bud blight of soybean [*Glycine max* (L.) Merr.], which is caused by the seedborne and sap-transmitted tobacco ringspot virus (TRSV), is a widely distributed, prevalent, and yield-reducing disease of this crop and of other leguminous plants (1, 10). Recently, we characterized TRSV in soybean roots and in root nodules of plants grown from TRSV-infected seed as well as from artificially inoculated plants (7). Tobacco ringspot virus also has been detected in meristematic tissue of root tips and in the inner root cap of bean (*Phaseolus vulgaris*) (5).

In the United States the importance of this disease varies with the amount of TRSV-infected seed planted and the subsequent dispersal of the virus. In continental China, the center for soybean domestication and source of several wild species of *Glycine,* budblight ranks with soybean mosaic and soybean stunt as one of the three most important virus diseases of soybeans (19); it also is present in other countries.

Research conducted thus far on diseases of soybean that interact with *Rhizobium japonicum* (Kirchn.) Buchanan has been, with a few exceptions (7, 15, 16, 20, 21), very limited. Studies of the interaction of soybean viruses and *R. japonicum* have dealt mainly with soybean mosaic virus (SMV) as it affects nodulation and total nitrogen content (21) and nodule infection (20). Similar research also has been reported on the Arhar mosaic of pigeon peas (18), enation mosaic of *Dolichos* (17), and other bean mosaics. We recently reported briefly on the effects of TRSV on N_2 fixation in soybeans (14).

Leghemoglobin (LH), the heme protein complex, is likely to function in the symbiotic fixation process by facilitating diffusion and regulation of O_2 for the respiration of the nitrogenase-containing *Rhizobium* bacteroids in the nodule (4, 6, 8, 24). Myoglobin has a similar function in the regulation of pO_2 in mammalian muscle cells (23, 24). In leguminous plants it has been shown that LH biosynthesis coincides with the onset of the symbiotic process in the young plant (3, 8, 23). It is apparent, however, that such an association becomes less pronounced as the plant ages.

We now present additional data on soybean infection

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by TRSV as it affects plant development, LH accumulation, and the efficiency of the endosymbiotic N_2-fixation process during the various stages of the growth cycle. Implications of the effect of this virus-bacteria interaction upon soybean production are discussed.

MATERIALS AND METHODS

Quantification of growth parameters.—Harosoy soybeans belonging in maturity group II were grown in pasteurized farm soil in 10.2- and 15.2-cm (4- and 6-inch) diameter pots in the greenhouse. A suspension of *Rhizobium japonicum* strain 110 (15) was used as seed inoculant. To prepare the inoculant *Rhizobium* suspension, the bacterium was grown in yeast-mannitol broth for 10-15 days and the cells were separated by centrifugation and resuspended in distilled, de-ionized water with a small amount of acacia gum to improve adherence to the seed. All inoculations were made at the rate of approximately 10^6 *Rhizobium* cells per seed.

Soybean plants were inoculated on the primary leaves with either purified TRSV-WS-1 or with TRSV-infected soybean or cowpea sap in 0.02 M phosphate buffer of pH 7.0. Noninfected control plants were grown from uniform seed harvested from symptomless and apparently virus-free Harosoy soybeans grown at Beltsville, Maryland. Plant growth response to virus infection was based on yields (fresh weight basis) of tops, roots, and nodules that were sampled during the seedling and prebloom, bloom and seed-set, bloom and early pod, pod-fill, and mature-pod stages. Yields given are means of four replicated pots (with four plants per pot) for each growth stage. In view of our recent findings (7), nodules collected from TRSV-infected plants are referred to as TRSV-infected nodules.

Quantification of leghemoglobin.—Nodule samples used for determination of LH content were collected intact from the same plants used for the determination of top and root yields and for N_2-fixation assays. Soybean LH was determined (22) as total cyanmethemoglobin (CMH) in milligrams per gram-nodule (fresh weight basis) with slight modification to suit our sample nodule tissue. This method is based on the oxidation of LH to methemoglobin and conversion to CMH with KCN. The absorption spectrum of CMH, expressing LH prepared from soybean nodule extracts, is very similar to that of standard CMH, prepared from blood hemoglobin (Standard Stock 525-18, Sigma Chemical Co., St. Louis, MO 63178), as shown in Fig. 1. The absorption peaks for CMH from both soybean and blood occur at a wavelength of 540 nm as determined with a Bausch and Lomb Spectronic-20 spectrophotometer. Actual soybean CMH concentration was determined by measuring the optical density at 540 nm and by comparing it with that of a standard CMH curve. The concentration of LH as CMH in the nodule samples are means of determinations from nodule tissue sampled for each growth stage.

Quantification of symbiotic N_2 fixation by acetylene reduction.—Reduction of acetylene (C_2H_2) to ethylene (C_2H_4) indicating nitrogenase-catalyzed N_2 fixation activity (8) of TRSV-infected and noninfected soybean plants was monitored on the same plants used for the determination of growth parameters and LH content. Nitrogen fixation rates determined by the C_2H_2 reduction assay (9) were expressed in micromoles of $C_2H_4 \cdot \text{plant}^{-1} \cdot \text{hr}^{-1}$ as well as $C_2H_4 \cdot \text{gram-nodule}^{-1} \cdot \text{hr}^{-1}$ (on a fresh-weight basis). Theoretically, C_2H_2 reduction rates per plant or per gram-nodule are equivalent to one-third of the actual N fixed in the symbiosis.

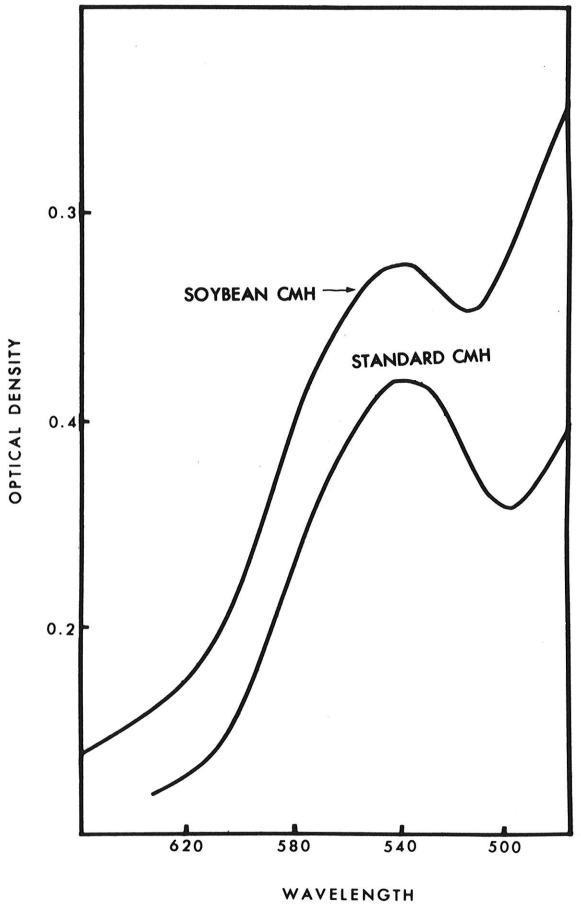

Fig. 1. Spectral absorbances at 540 nm of standard cyanmethemoglobin (CMH) prepared from human hemoglobin (Standard Stock 525-18 Sigma Chemical Co., St. Louis, MO 63178) at a concentration of 4 mg/10 ml, and of soybean CMH, expressing leghemoglobin (LH), extracted from soybean nodules.

RESULTS

Effect of TRSV on plant growth and nodulation.—Severe budblight was readily induced 8-10 days after inoculation of the primary leaves of Harosoy soybean in the greenhouse. Axillary adventitious branches that developed subsequently also underwent budblight in addition to the other symptoms of the syndrome. Fig. 2-(A to C) show that top, root, and nodule yields of *Rhizobium*-inoculated, TRSV-infected plants were significantly lower than those of noninfected plants at each of the five growth stages. Except for rudimentary nodule initials which occasionally were discernible on the

tap root of TRSV-infected plants, further differentiation of these structures into functional nodules failed to occur until these TRSV-infected plants were approximately 47 days old and were in the bloom and early pod stage. Following the non-nodulating period, nodules began to form and continued forming almost up to the end of the growth cycle. In contrast, well developed, LH-containing nodules were already present on the tap root and seconday roots at the time the noninfected plants emerged. Mean nodule weights for TRSV-infected 47-, 62-, and 77-day-old plants, respectively, in bloom and early pod, pod-fill, and mature-pod stages decreased 85, 67, and 67%, respectively, compared to noninfected plants (Fig. 2-C). Extremely low nodule development also occurred in field plantings of TRSV-infected, *Rhizobium*-inoculated Harosoy soybeans at Beltsville, Maryland (Orellana, *unpublished*).

Effect of tobacco ringspot virus on leghemoglobin accumulation.—Mean LH contents determined as CMH in milligrams per gram-nodule (fresh weight basis) for nodule samples from 47-, 62-, and 77-day-old TRSV-infected plants from 27-, 36-, 47-, 62-, and 77-day-old noninfected plants are shown in Fig. 3. Even though LH content of infected nodules from 47-day-old plants in the bloom and early pod stage decreased 3% compared to noninfected plants, there was a considerable increase of 33 and 25% in LH content during the subsequent pod-fill and mature-pod stages. These increased LH levels as the plant aged corresponded to decreased nodule weights of 67 and 67% compared to those of noninfected control

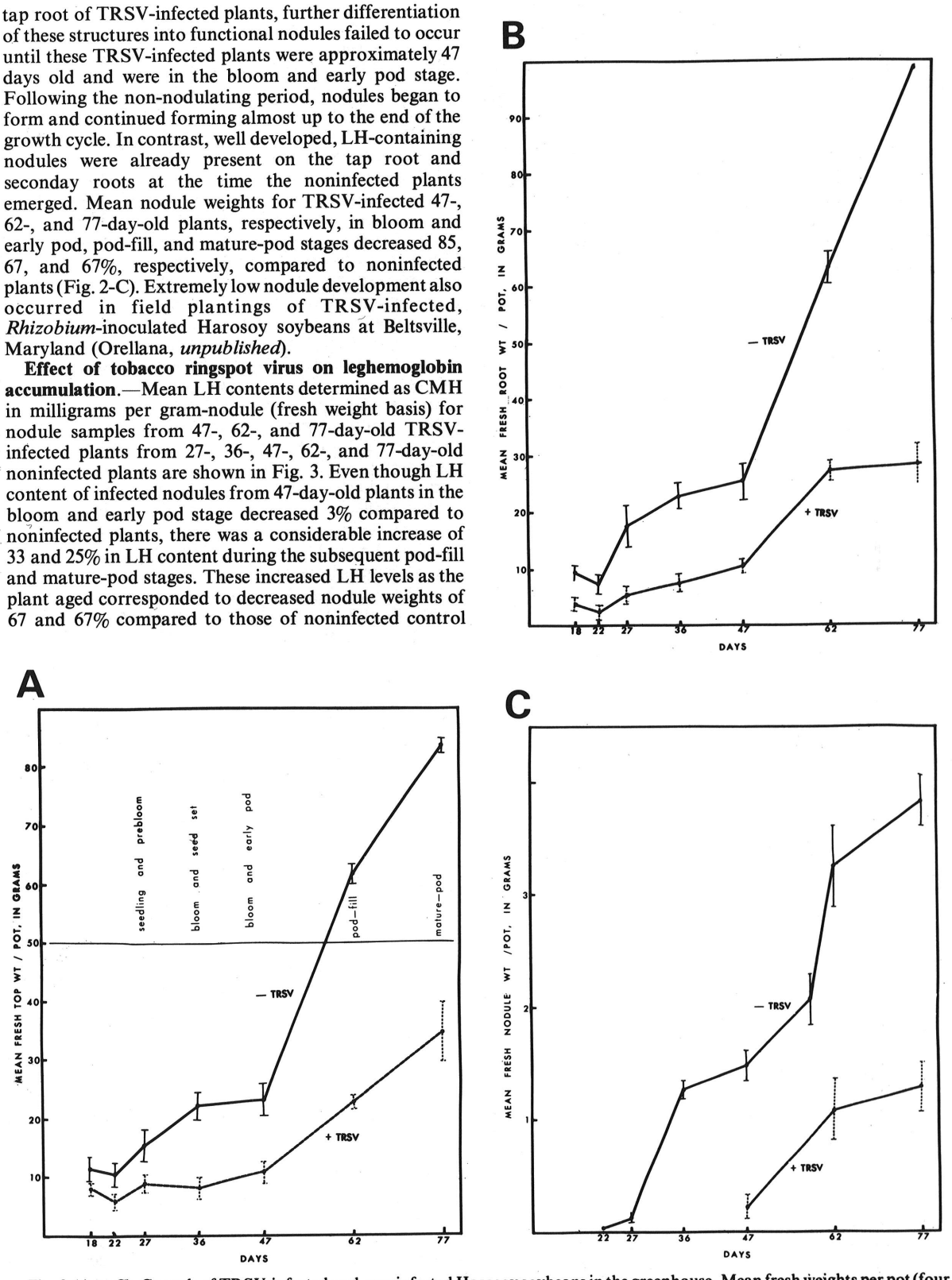

Fig. 2-(A to C). Growth of TRSV-infected and -noninfected Harosoy soybeans in the greenhouse. Mean fresh weights per pot (four plants/pot), in grams ± the standard deviation of the mean were recorded for each of the five growth stages durig the 77-day cycle for plant **A**) tops, **B**) roots, and **C**) nodules.

plants. These experiments further demonstrated that the LH concentration of nodules from TRSV-infected plants was higher and peaked later and the plants remained green and vegetative for approximately 2-3 wk longer than noninfected plants. Apparently this increased, although late, symbiotic activity may have been too late to increase root, nodule, top, and seed yield. Delayed plant senescence also was observed in TRSV-infected soybean plantings at Beltsville, Maryland.

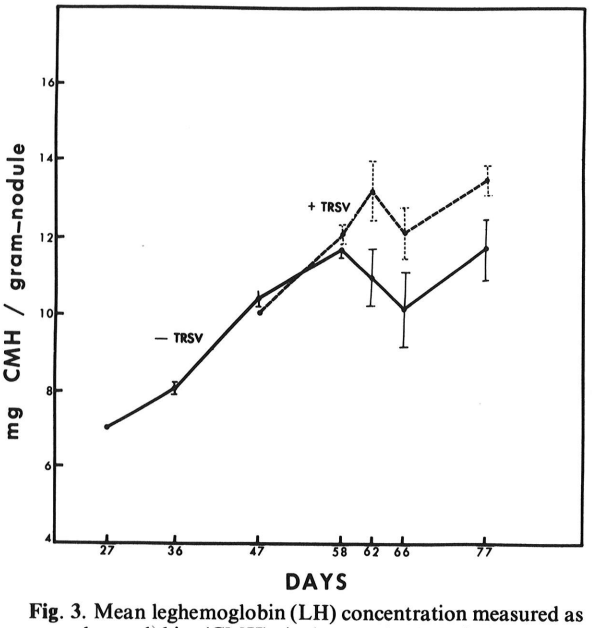

Fig. 3. Mean leghemoglobin (LH) concentration measured as cyanmethemoglobin (CMH) ± the standard deviation of the mean in nodules from TRSV-infected and -noninfected soybean plants grown in the greenhouse. Values are given in milligrams of CMH/gram-nodule (fresh weight basis) for each growth stage.

Effect of tobacco ringspot virus on nitrogen fixation.—Symbiotic N_2 fixation activity expressed in micromoles $C_2H_4 \cdot$ gram-nodule$^{-1} \cdot hr^{-1}$ and $C_2H_4 \cdot$ plant$^{-1} \cdot hr^{-1}$, is shown, respectively, in Fig. 4 and 5. Because TRSV-infected plants nearly failed to nodulate during the first 47 days of the growth cycle, no attempt was made to monit or N_2 fixation during that time. As shown in Fig. 4, C_2H_2 reduction to C_2H_4 rates on a gram-nodule basis for 47-, 62-, and 77-day-old infected plants increased 30, 99, and 57% relative to noninfected plants even though these increases never reached the total amount of the healthy plants. Except for the slight decrease of 3% in LH content of infected nodules from plants in bloom and early pod, the late N_2 fixation activity during the subsequent pod-fill and mature-pod stages increased with the increased LH content during these late growth stages. However, when C_2H_2 reduction to C_2H_4 rates were calculated on a plant basis as shown in Fig. 5, the reduction rates for infected plants decreased consistently 81, 41, and 23% from the third to the fifth growth stage, as compared to that of noninfected plants. Apparently this was due to the reduction of nodule development brought about by the virus during the early growth stage of infected plants.

These experiments further demonstrated that, in TRSV-infected plants, the LH concentration in nodules was higher and peaked later and the plants remained green and vegetative for about 2-3 wk longer than in noninfected control plants (Fig. 3). It is likely, therefore, that this increased activity may have been too late to increase root, nodule, top, and seed yields.

Statistical analysis of the interactions between LH

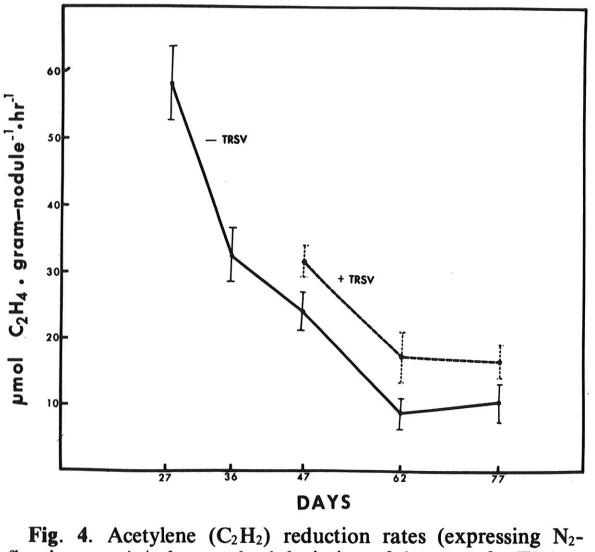

Fig. 4. Acetylene (C_2H_2) reduction rates (expressing N_2-fixation rates) ± the standard deviation of the mean for TRSV-infected and -noninfected soybean plants grown in the greenhouse. Nitrogen fixation rates are expressed on a fresh weight basis, in micromoles of $C_2H_4 \cdot$ gram-nodule$^{-1} \cdot hr^{-1}$ for each growth stage.

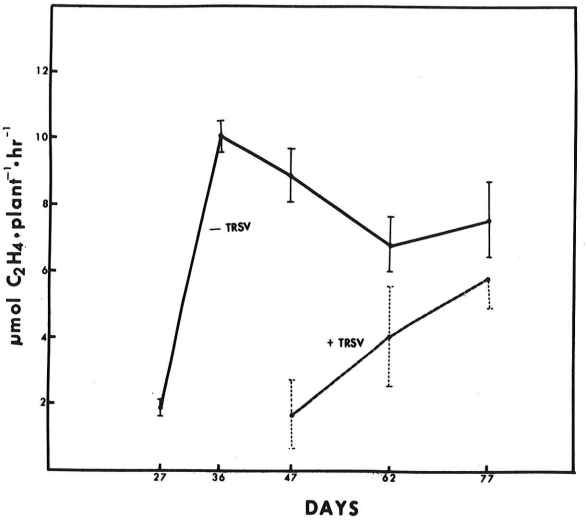

Fig. 5. Acetylene (C_2H_2) reduction rates (expressing N_2-fixation activity) ± the standard deviation of the mean for TRSV-infected and -noninfected soybean plants grown in the greenhouse. Nitrogen fixation rates are expressed in micromoles of $C_2H_4 \cdot$ plant$^{-1} \cdot hr^{-1}$ for each growth stage.

concentration and N_2-fixation rates indicated highly significant negative correlations, $r = -0.9900$ and $r = -0.8905$, for the TRSV-infected and noninfected plants during the experimental growth cycle. The linear regressions corresponding to these correlation coefficients are shown in Fig. 6. This statistical study was verified by computer analysis at the Data Systems Application Analyses, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD.

DISCUSSION

The most striking feature demonstrated in this study of the TRSV-*Rhizobium* interaction in the soybean plant was the nearly complete, although transitory, inhibition of root-to-nodule tissue differentiation that caused a suppression of nodule development and of symbiotic N_2-fixation activity which lasted approximately 6 wk until the plants were in the bloom and early pod stage. This period of nodule suppression apparently was associated with an energy and nutrient deficit which was brought about by early and severe budblight and dysfunction of older leaves.

After the nodules began to form on the maturing TRSV-infected plants, LH content was found to be significantly higher than that of nodules of maturing healthy plants. This late increase in LH content was evident as the infected plants approached blooming, and LH content reached its maximum activity about 3 wk later than in noninfected plants. This maximum LH activity was followed by a drop and then by an increase in LH content as the plants matured. The drop in LH content may have been due to the low LH content in newly formed nodules that developed on secondary roots of TRSV-infected plants. The possibility that this altered nodulation pattern and enhanced LH concentration could have been associated with increased virus multiplication and protein synthesis is an intriguing question that requires further studies of the metabolism of virus-infected plants. Although changes in cell morphology, surface membrane activity, cell division, immune response, and other cell functions have been intensely investigated in virus-infected animal cells by several investigators (2), comparable studies on virus-infected plant cells are lacking. If changes of such magnitude would occur in TRSV-infected rhizobial and nodule cells, it is conceivable that, for example, the pO_2 regulating capacity of LH that is required for efficient nitrogenase activity in the bacteroids, the reduction of N_2 to NH_3, and other functions (11, 23) that are paramount to the symbiotic process would be affected. It is possible also that the virus may interfere with entry of the *Rhizobium* infection thread into the root hair and thus prevent the establishment of the symbiosis. Among plant viruses, TMV has been shown (13) to accumulate in large amounts in root hairs of TMV-infected tobacco. Whether or not TRSV would accumulate in high amounts in soybean root hairs has not been determined.

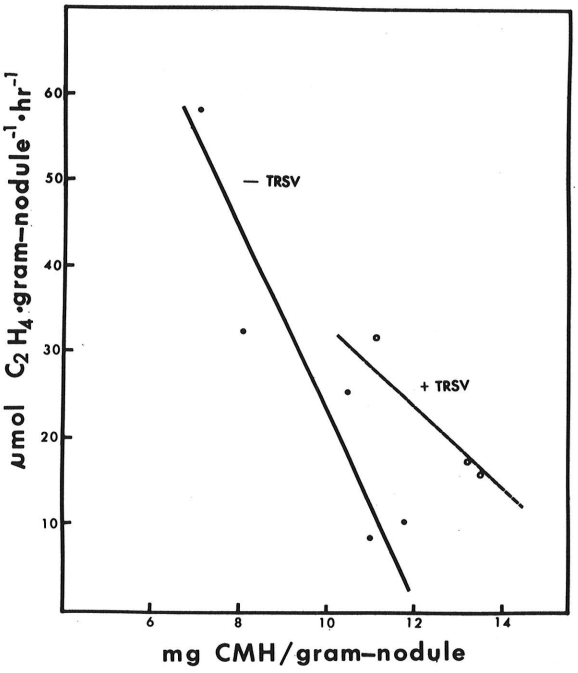

Fig. 6. Relationship between leghemoglobin (LH) and acetylene reduction rates (expressing N_2-fixation activity) of TRSV-infected and noninfected soybean plants. Data used for plotting the linear regression of noninfected plants correspond to the five growth stages of the growth cycle. Data of the infected plants correspond to three growth stages only as N_2 fixation activity in these plants, during the first two growth stages, was suppressed.

Because a greater number of observations, besides those made at the five growth stages, perhaps would have been desirable, caution should be taken in drawing final conclusions concerning the relationship between these metabolic parameters; i.e., LH concentrations and N_2 fixation in TRSV-infected and noninfected plants.

Even though the carbon metabolism of *Pisum sativum*, a legume that in many respects is akin to the soybean, has been investigated (12), no attempt was made in the present investigation to measure photosynthetically fixed C in soybean either in the presence or in the absence of TRSV. Studies are needed therefore, to elucidate the effects that this and other virus diseases may exert on photosynthetically fixed C and symbiotically fixed N_2 in soybeans.

In conclusion, the results of the present investigation demonstrate that soybean budblight disease severely delays nodulation and interferes with the efficiency of the N_2 fixation process. The consequences of delayed nodulation, impairment of LH accumulation and N_2 fixation, in spite of the greater accumulation of LH which occurred in TRSV-infected nodules than in noninfected nodules as the plants aged, would be too late under field conditions to promote yield. Effective exclusion of the budblight virus, or reduction of the inoculum potential from soybean plantings by means of disease resistance breeding, sanitation, or by methods of viral inactivation would contribute to the management of the disease and the attainment of higher seed yields. It is possible that attainment of greater soybean yields also may depend on exclusion of less severe diseases which nevertheless elicit cellular disturbances in the *Rhizobium* root nodule. This concept is supported by our earlier demonstration of

Rhizoctonia-induced nodule cell dysfunction and reduced N_2 fixation (15, 16), and as shown in the present investigation, by significant impairment of the *Rhizobium*-soybean symbiotic system by TRSV.

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