Some Factors Affecting the Nodulation and Nodule Efficiency in Soybeans Infected by Soybean Mosaic Virus

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Iowa Agriculture and Home Economics Experiment Station Journal Paper No. J-6534, Ames, Project No. 1628.
Accepted for publication 15 June 1970.

ABSTRACT

Total nitrogen in nodules of soybeans infected by soybean mosaic virus (SMV) was consistently higher, and nodule weight was consistently lower, than in comparable healthy plants at different temperatures, daylengths, and growth stages.

Differences in nodule weight between SMV-infected and healthy soybeans were small at 15.5 C, but increased with increasing temperature (to 26.5 C). Similarly, differences in nodule weight between SMV-infected and healthy soybeans were small at a short (6 hr) daylength, but increased at longer daylengths (10 and 14 hr). When inoculated with *Rhizobium japonicum* at different growth stages, healthy soybeans were always more susceptible to *Rhizobium* infection than were SMV-infected plants.

Reduced susceptibility to *R. japonicum*, decreased leghemoglobin, and increased total nitrogen in SMV-infected soybean nodules suggests that nodules on SMV-infected plants were less efficient than nodules on healthy plants. Phytopathology 60:1653-1656.

Yield and plant-wt reduction in soybeans infected by soybean mosaic virus (SMV) have been attributed both to photosynthetic reduction (1, 3, 4, 12) and to reduced nodulation (7, 13). Although reduction of nodule size and numbers has been shown in SMV-infected soybeans, we do not know if the efficiency of N fixation by these nodules has been impaired. Variations in size and numbers of nodules may not be important factors in plant growth without knowing their efficiency in N fixation. Increased efficiency could offset any deleterious effects of reduction of size and number of nodules. Efficiency of N fixation of SMV-infected soybean nodules may be influenced by environmental factors, such as temp, daylength, and the growth stage of the soybean plant.

The object of this study is to assess N-fixation efficiency in nodules of SMV-infected soybeans and to determine how environment influences their interaction.

MATERIALS AND METHODS.—Soybean (*Glycine max* [L.] Merr. 'Bansei') and soybean mosaic virus isolate 'O' (SMV) (9) were used in all experiments. Nodulation was induced by *Rhizobium japonicum* (Kirchner) Buchanan.

Temperature and daylength.—All plantings were made in nonsterilized sand:peat:loam (1:1:2) soil mixture. The soil used was previously tested for presence of Rhizobia. Two seeds were planted in 72 6-inch pots for each experiment. The greenhouse temp was 24 ± 3 C. SMV inoculum was expressed crude sap from SMV-infected leaves diluted ×10 with 0.01 M phosphate buffer, pH 7.0. Inoculum was rubbed on leaves previously dusted with 600-mesh Carborundum. Seedlings in 36 pots were inoculated at the unifoliolate stage 2 weeks after seeding, and 36 pots served as controls. Controls were plants rubbed with phosphate buffer and Carborundum to simulate inoculation. Leaves were rinsed with tap water immediately after inoculation. Plants were left in the greenhouse 1 week, then infected, and control plants were divided into three groups each and placed in three Percival PGC-78 growth chambers.

Temperatures were maintained at 15.5, 21, or 26.5 ± 0.5 C, and each chamber was programmed for 10-hr nights and 14-hr days, with a fluorescent light intensity of 1,400 ft-c at bench level. Nodules were harvested 2.5 months after seeding.

For daylength experiments, the chambers were adjusted to 6-, 10-, or 14-hr days; all were maintained at 21 C. Nodules were harvested 2 months after seeding. The method of nodule harvest has been described (13). All experiments were repeated at least once; the data were comparable.

Host susceptibility.—Seed were surface-sterilized for 30 sec in 0.5% sodium hypochlorite, rinsed twice with distilled water, soaked 5 min in 70% ethyl alcohol, then rinsed 3 times with distilled water. Five seeds were planted/6-inch pot, in a commercial vermiculite and perlite mixture (1:1) previously sterilized at 12 psi, 100 C, for 6 hr. Tap water was used in watering until seedlings emerged. Thereafter, watering was with modified Hoagland's solution (6). Potted plants were kept in a greenhouse with supplemental cool-white fluorescent lights which provided at least 800 ft-c, in addition to sunlight for a total 14-hr daylength.

Uniform seedlings, 144 pots, were selected and divided equally for four treatments. Treatments were (i) nontreated control; (ii) seedlings inoculated with *R. japonicum* only; (iii) seedlings inoculated with SMV alone; and (iv) seedlings inoculated with both SMV and *R. japonicum*.

Plants were inoculated with SMV at the unifoliolate leaf stage. Each treatment was divided into six groups, six pots/group. *Rhizobium japonicum* inoculation was started 2 days after SMV inoculation for the first group, and an additional group was inoculated after each subsequent 5-day interval. Beltsville strains 110 and 123 of *R. japonicum* (obtained from L. R. Frederick, Iowa State Univ.) were used to inoculate each group, three pots/strain. All controls were divided similarly and were placed beside treated groups.

Rhizobial inocula were grown at 22 C on a shaker for 2 days in yeast-mannitol broth (5). This was diluted

×10 with Hoagland's solution immediately before use. The test solution contained 10^6 bacteria/ml. Inoculation was made by applying 50 ml of this inoculum to each pot. Nodules were harvested separately from each treatment 1 month after the last rhizobial inoculation, approximately 2.5 months after seeding.

Leghemoglobin and total N determination.—Leghemoglobin content in nodules was measured according to an addendum leghemoglobin determination (I. E. Anderson, Iowa State Univ., *personal communication*). All nodule samples were frozen before leghemoglobin extraction. Four replicate samples were measured for each treatment. One g of nodules was homogenized in 5 ml of 0.1 N KOH and centrifuged 10 min at 12,000 *g*, and a sample of 1.5 ml of supernatant was mixed with 1 ml of water and 0.5 ml of 5 N KOH; 0.1 g of $Na_2S_2O_4$ was then added for reduction, and OD of leghemoglobin was determined at 537, 557, and 577 mμ wavelengths 10 min after mixing. The OD for leghemoglobin was calculated using the formula OD $557 - \frac{1}{2}$ ($OD_{537} + OD_{577}$).

Total N content in nodules was determined in a Technicon Nitrogen auto-analyzer (determinations made at OARDC, Wooster, Ohio). Two hundred-fifty mg of dried, ground nodule material was thoroughly digested in 7 ml of 0.3 M H_2SeO_3 in concd H_2SO_4, and 100 ml distilled H_2O added and filtered through Whatman No. 1 filter paper. A 45-ml aliquot was mixed with an appropriate amount of water, NaOH (2.8%), alkaline phenol (1.9% phenol in 20% NaOH), and sodium hypochlorite (5% chlorine solution). The resultant solution was measured in a spectrophotometer at 630 mμ.

RESULTS.—*Temperature.*—Healthy soybean plants had slightly more nodules and higher fresh nodule wt than did SMV-infected plants at 15.5 C; at 21 and 26.5 C, however, a greater increase in both number and fresh wt of nodules was observed in healthy soybean plants compared with SMV-diseased ones (Table 1). For example, nodule wt in healthy plants at 21 C was 0.570 g and 1.150 g at 26.5 C, a 100% increase, while nodule wt increased very little (2.1%) in SMV-diseased plants at these temp (Table 1). These results correlated positively with the symptom severity of SMV-diseased plants at various temp. Mosaic disease symptoms were inconspicuous at 15.5 C (11), but symptom severity increased with increase in temp to 26.5 C.

Reduction of nodule wt in diseased plants was greater than reduction of plant fresh wt, as previously reported (13). The difference was most obvious at 26.5 C, where a 68% reduction in nodule wt corresponded to a 15% reduction in plant fresh wt (Table 1).

Daylength.—Although soybeans for daylength experiments were harvested 2 weeks earlier than those for temp experiments, the daylength effect on nodulation of healthy and SMV-diseased soybeans was similar to the temp effect; i.e., directly proportional. Daylength, however, seemed to affect nodule number less and fresh wt of nodules more. For example, when comparing 10- and 14-hr daylengths, healthy and diseased plants at 14 hr had 20 and 40% more nodules, respectively. Nodule wt for similar treatments were increased 166 and 236%, respectively.

Host susceptibility.—When healthy and SMV-infected soybeans of different growth stages were inoculated with either *R. japonicum* strain 110 or 123, healthy soybeans produced considerably greater numbers of nodules than did SMV-diseased soybeans. Therefore, the healthy plants apparently were more susceptible to rhizobial inoculation than were the SMV-diseased plants of the same growth stage.

The most susceptible stage of soybean to rhizobial infection was probably the unifoliolate stage, about 14-20 days after seeding. Thereafter, susceptibility declined sharply. Susceptibility to rhizobial infection in SMV-diseased soybeans was greatest at the most virus-susceptible growth stage of soybeans. Host susceptibility differences between healthy and SMV-diseased soybeans gradually narrowed as the plants became older. For example, when healthy and SMV-diseased soybeans were inoculated with strain 110 7 days after SMV infection, they produced 408 and 101 nodules, respectively. This figure represents a decrease of 60% in susceptibility to rhizobial inoculation in SMV-diseased soybeans at the unifoliolate stage. On the other hand, if *R. japonicum* was inoculated 17 and 22 days after

TABLE 1. Effect of temp and daylength on number, wt, and nitrogen content of nodules from healthy and soybean mosaic virus-infected soybeans

	Total N in nodules		No. and fresh wt of nodules/plant				Plant fresh wt/plant	
	Healthy	Diseased	Healthy		Diseased		Healthy	Diseased
Temp[a] C	%	%	*no.*	*g*	*no.*	*g*	*g*	*g*
15.5	3.80[b]	4.05	12.1	.25	10.5	.23	4.7	4.3
21	4.00	4.35	34.4	.57	19.2	.30	7.9	5.5
26.5	4.00	4.60	63.5	1.15	23.2	.36	11.3	9.6
Day length[a] (hr)								
6	3.80	4.15	18.0	.10	16.1	.07	5.2	3.3
10	3.60	3.72	30.2	.28	17.5	.10	8.5	5.8
14	3.55	3.60	36.1	.76	20.4	.35	12.5	9.7

[a] Nodules were harvested 2.5 months after seeding for the temp experiments, 2 months after seeding for daylength experiments.

[b] All figures are an average of four replicates, 10 plants/replicate.

TABLE 2. Effect of age of healthy and soybean mosaic virus (SMV)-infected soybean on susceptibility to *Rhizobium japonicum* and on N content of nodules

Rhizobial inoculation (days after SMV-inoculation)	Total N in nodules		Nodule production/plant					
			Healthy inoculated with Rhizobia strain		Healthy not inoculated with Rhizobia	SMV-infected inoculated with Rhizobia strain		SMV-infected not inoculated with Rhizobia
	Healthy	SMV-infected	110	123		110	123	
	%	%	*no.*	*no.*	*no.*	*no.*	*no.*	*no.*
2[a]	4.50[b]	4.50	21.4	23.0	1.2	7.3	3.2	0.7
7	4.75	5.00	40.8	16.0	0.7	10.1	2.6	0.5
12	4.50	4.60	16.0	8.5	0.6	6.8	2.5	1.2
17	4.00	4.20	6.2	5.5	1.0	4.3	1.8	0.4
22	3.80	4.15	3.8	2.8	0.8	3.0	1.0	0.5
27	3.50	3.80	2.6	2.0	0.9	1.7	0.8	0.7

[a] Time for first rhizobial inoculation (zero time) which was 2 days after SMV inoculation and 16 days after seeding.
[b] All figures are an average of four replicates, 10 plants/replicate.

virus infection, SMV-infected plants were 22 and 21% less susceptible than comparable healthy ones, respectively (Table 2).

Rhizobium strain 110 was more virulent than strain 123 to both healthy and SMV-diseased soybeans. The most susceptible growth stage of soybeans for strain 110 was the unifoliolate stage, 21 days after seeding; for strain 123, the late cotyledon stage was most susceptible, approx 16 days after seeding. A few nodules developed on healthy and SMV-infected control plants even though precautions (autoclaving vermiculite and pots, and surface-sterilizing seed) were taken (Table 2).

SMV was isolated as readily from nodule tissue of SMV-infected soybeans as from their roots or leaves.

Leghemoglobin and total N determination.—A separate experiment was conducted to determine the relationship of leghemoglobin and total N in nodules, as leghemoglobin is believed responsible for the pink color of nodules and is related to the activity of N-fixing activity in nodules (8). Nodules of healthy plants had higher leghemoglobin contents than did those of SMV-diseased nodules (Fig. 1).

Total N content in nodules was determined for all experiments. In the temp experiment (Table 1), total N contents in nodules of healthy soybeans were lower than in those of SMV-diseased ones. Total N increased with increasing temp, although it leveled off in healthy nodules after reaching the 4% level while that in diseased nodules continued to increase (Table 1). The effect of daylength on total N in nodules was similar to the temp effect, since nodules from SMV-diseased soybeans contained higher total N than did those of healthy soybeans. However, total N in nodules of both healthy and SMV-diseased soybeans decreased slightly with increasing daylength (6-14 hr) (Table 1).

Similarly, higher total N in nodules of SMV-diseased plants was measured in the host-susceptibility experiment in which nodules of different ages were available. In all instances, nodules of SMV-diseased soybeans had higher total N contents than did those of comparable healthy soybeans (Table 2). Total N content generally increased with increasing nodule age in nodules from both healthy and SMV-diseased soybeans. These results compared favorably to those obtained for total N in the daylength experiments. For example, total N in nodules from 21 C, 14-hr daylength, in the daylength experiment, should theoretically be about the same as the total N in nodules from 21 C, 14-hr daylength in the temp experiment. The total N in the daylength experiment was lower, however, because the nodules were harvested 2 weeks earlier than in the temp experiment.

DISCUSSION.—Joshi et al. (7) found that healthy white clover (*Trifolium repens*) produced large pink root nodules, but that phyllody-infected clover produced mainly small white nodules characteristic of those with a reduced effectiveness in N fixation. Tu et al. (13), however, did not observe such striking color differences in nodules of healthy and SMV-infected soybeans. Although not visible, considerably less leghemoglobin was detected in nodules of SMV-infected plants early in infection. Leghemoglobin apparently has no direct role in the N-fixation reaction (10). Total N content in

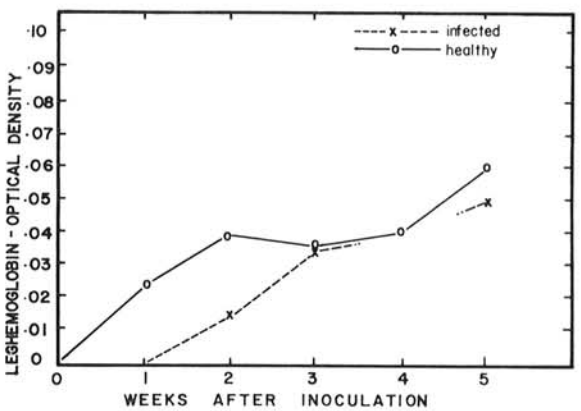

Fig. 1. Relative leghemoglobin content in root nodules of healthy and soybean mosaic virus (SMV)-infected soybeans. SMV inoculation was made 2 weeks after seeding. The datum point for week 4 for SMV-infected soybeans was invalid because of technical difficulties. Data are averages of four replications.

nodules generally is believed most reliable for determining the effectiveness of nodules in healthy soybeans. Since nodules of SMV-infected soybean contained higher total N than did their healthy counterparts, however, this increase, accompanied by a decrease in leghemoglobin, indicates a metabolic disturbance caused by the virus, probably indirect.

Previously, we reported that SMV-infected soybean leaves were accompanied by increases in free amino acids, compared with healthy plants (14). Thus, the increased free amino acids in SMV-infected plants may occur also in nodules and be reflected as more total N. Unless previously fixed N was effectively used and (or) transported elsewhere, the presence of excess nitrogenous compounds could alter the C:N ratio which might inhibit the normal rate of N fixation. This could result also in decreased leghemoglobin synthesis. Application of N fertilizer inhibited nodulation and nodule wt increase (13, 15, 16). Thus, increased total N content in root nodules may be responsible for the leghemoglobin decrease in nodules of SMV-diseased soybeans.

Nodule production in healthy plants seems more responsive to temp than that in diseased plants. Temperature- and daylength-effect data (Table 1) help to substantiate our explanation of the relationship of total N and leghemoglobin.

SMV-diseased soybeans seemed to respond to an increase in daylength based on nodule wt. Although increasing daylength did not increase symptom severity in SMV-diseased soybean, it increased plant growth rate somewhat, probably due to increased photosynthesis. This increased plant growth of SMV-infected soybean, plus decreasing total N in nodules with increasing daylength, suggests that increased daylength may stimulate utilization of fixed N which would tend to increase N-fixation in nodules. Our finding agrees with Van Schreven's (15) results that shading pea plants reduced nodule size, and that subsequent spray applications of sucrose improved nodule size and efficiency of N-fixation. Total N in nodules of healthy soybeans also decreased with increasing daylength, but the decrease was not as marked as that in SMV-infected soybeans.

SMV symptoms increase in severity with increasing temp. Similarly, total N continues to increase (accumulate) in nodules on SMV-diseased soybeans with increasing temp, compared with those on healthy soybeans.

Two explanations for higher total N in nodules of SMV-infected soybeans may be valid. (i) The rate and amount of N fixation by *R. japonicum* may be influenced directly by SMV infection, but our evidence does not allow us to draw this conclusion. (ii) The utilization of nitrogenous compounds fixed in nodules by *R. japonicum* may be inefficient because of the added stress on the biological system of supporting SMV replication. Amino acid increases correlated with symptom severity occur in SMV-diseased leaves (2, 14), suggesting that a total N increase could be expected also in SMV-infected roots. Even more specifically, the total N increase in nodules may reflect only the sum of expected increase in free amino acids in SMV-diseased tissues, plus the accumulation of N that *R. japonicum* is fixing at a normal rate, but which is not being metabolized efficiently or by the usual pathways of the healthy plant.

Therefore, we believe that increasing total N contents, plus decreasing leghemoglobin contents, are signs of accumulation due to inefficient N utilization in SMV-infected soybeans.

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