Specific Effects of Soybean Mosaic Virus on Total N, Ureide-N, and Symbiotic N_2-Fixation Activity in *Glycine max* and *G. soja*

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ABSTRACT

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Plants of cultivated soybeans (*Glycine max* 'Hill' and 'Essex') and two introductions of wild annual soybean, *G. soja* PI 424-005 and PI 378-693-B, were studied in the greenhouse for their response to soybean mosaic virus. Significant decreases in top and nodule weights had occurred after 53 days in plants inoculated in the primary leaf stage with the Beltsville isolate of soybean mosaic virus (SMV-B). Plants of *G. soja* infected with SMV-B had significantly higher total N and ureide-N contents than the controls. Plants of *G. max* cultivar Hill showed no significant differences for these two parameters, while total N increased significantly in infected plants of cultivar Essex. N_2-fixation activity, measured by the acetylene reduction assay, decreased significantly in all genotypes except Hill. Measurements of nodule-specific nitrogenase activity indicated that nodules from plants infected with SMV-B of Hill and PI 378-693-B fixed N_2 at rates exceeding those of the uninoculated controls. The presence of SMV-B was detected by ELISA in inoculated plants at harvest. Leaves developed higher virus concentrations than did nodules, and there was no correlation between foliar virus titer and symptom severity ratings. Symptoms were most severe on *G. soja* PI 424-005, less severe on *G. max* cultivar Essex and *G. soja* PI 378-693-B, and least severe on *G. max* cultivar Hill.

Additional key words: legumes, *Rhizobium japonicum*, wild soybean germplasm.

The pathological and physiological effects of soybean mosaic virus (SMV) (1) on growth, nodulation caused by *Rhizobium japonicum* (Kirch.) Buchanan, and the symbiotic N_2-fixation process in cultivated soybean (*Glycine max* (L.) Merr.) plants are of considerable economic importance. Fixed N_2 has been estimated to account for 23–75% of the plant's total nitrogen needs (5,10). Therefore, it seems likely that viral or other pathogenic interference in the fixation of atmospheric N_2 by the nodule bacterium, *R. japonicum*, would affect crop yield, especially when inefficient *R. japonicum* strains are used.

SMV translocation from the infected soybean leaves to the root-nodules was reported (20) to increase total nodule N content and to reduce nodule weight and leghemoglobin content, which caused the SMV-infected nodules to be less efficient than healthy ones. Also, it has been shown (21,22) that the total foliar free amino acid content and ammonia increased while amounts of the individual amino acids varied depending on the SMV isolate. In support of these findings, other soybean viruses, eg, tobacco ringspot virus (TRSV) (17), bean pod mottle virus (BPMV) (R. G. Orellana and S. L. Reynolds, *unpublished*), and bean yellow mosaic virus (BYMV) (16) also translocate readily from the leaves to the nodules and significantly affect nodulation and N_2-fixation activity. So far as we are aware, the effect of SMV on nodule-specific activity for functional nitrogenase in *G. max* or the wild annual soybean, *G. soja* Sieb. & Zucc., has not been investigated.

Because previous investigations of the effects of SMV infections on soybean have dealt only with the cultivated soybean (*G. max*), the present research was designed to determine the effects of SMV on total and ureide-N levels, N_2-fixation, and nodule-specific activity for both the cultivated soybean *G. max* and *G. soja*, and to investigate representative genotypes of wild soybean germplasm as potential sources of SMV resistance. A preliminary report on this research has been published (18).

MATERIALS AND METHODS

Soybean plants and inoculation with *Rhizobium*. Genotypes of *G. max* used in this investigation were the commercial cultivars Hill and Essex, both belonging in maturity group (MG) V (11) and two introductions of *G. soja* PI 424-005 and PI 378-693-B in MGs IV and VII, respectively. Soybean seeds were surface-disinfected with 50% ethyl alcohol for 0.5 min, rinsed twice with sterile distilled water, and planted in a fertilizer-free pasteurized loam soil and perlite mixture (6:1, v/v), pH 6.3, in 33 × 66.5 cm Speedling flats (Speedling Mfg., Inc., Sun City, FL 33586). This soil mixture was used for all treatments. At the time of planting, each seed was treated with 1 ml of a fresh yeast-mannitol broth culture of *R. japonicum* (USDA strain IIb-110) (19). All tests were conducted in fall and winter in a greenhouse with day and night ambient temperatures regulated to 23 ± 3 and 21 ± 3 C, respectively. Plants were grown under natural day conditions supplemented with approximately 1,500 lx provided by 200 W incandescent bulbs from 0600 to 1800 hours.

Virus source and characterization. The SMV isolate was obtained from a single soybean plant collected at an experimental plot at the Agricultural Research Center, U.S. Department of Agriculture, Beltsville, MD. Identification of this isolate was based on: a narrow host range and disease reactions of mechanically inoculated SMV indicator *G. max* hosts (3) and *Phaseolus vulgaris* L. 'Top Crop' (14); reaction with SMV antiserum in the enzyme-linked immunosorbent assay (ELISA) (4); and electron microscopy of leaf-dip and purified virus preparations. Pending further characterization, this isolate is designated SMV, Beltsville isolate

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(SMV-B).

Virus inoculations. SMV-B inoculum was prepared by grinding young, systemically infected leaves of cultivar Essex in 0.01 M phosphate buffer (pH 7.0) (1:10, w/v) ratio. Seedling plants of Essex, Hill, PI 424-005, and PI 378-693-B in the unifoliate VI stage (6) of the plant's vegetative development were mechanically inoculated with the virus using the carborundum gauze-pad method. Eight days after inoculation and after mosaic symptoms had developed on the expanding trifoliates, nine plants in three replicates (three plants per replicate) were transplanted into individual plastic pots, 12.5 cm in diameter, observed periodically for symptoms, and rated for severity. A fourth replicate was used as a source of tissue for serological assay. The same number of uninoculated plants served as controls.

Determination of top and nodule weights. Plant tops (stems, leaves, and pods) in the pod-fill stage (R5 stage of the plant's reproductive development) in three replicates per genotype were harvested 60 days after planting by cutting them at the first node. The tops were dehydrated at 70 C for 48 hr, ground in a laboratory mill fitted with a 0.5-mm (40-mesh) screen, and stored at −20 C. The nodules were detached from the roots, rinsed, and dried for a few minutes at room temperature and weighed.

Determination of functional parameters. Total N was determined by the Kjeldahl method in composite dried plant top samples consisting of three replicates (three plants per replicate) for each uninoculated and SMV-B-inoculated cultivars and plant introductions. Ureide-N levels were determined by acid hydrolysis of allantoin and allantoic acid to glyoxylic acid, which was measured spectrometrically following reaction with phenylhydrazide hydrochloride and potassium ferricyanide (23). N_2-fixation activity for excised roots of SMV-B-inoculated and uninoculated plants was determined by the acetylene reduction assay (9), which measures the rate of nitrogenase-catalyzed reduction of acetylene (C_2H_2) to ethylene (C_2H_4). Nodule specific activity for nitrogenase was calculated as micromoles C_2H_4 $g \cdot nodule^{-1} \cdot hr^{-1}$.

Serological assay of SMV-B. Concentration of SMV-B in soybean leaves and nodules was estimated by ELISA 55 days after inoculation. Young expanded trifoliates and nodules were collected from three SMV-B infected and three virus-free control plants for each genotype. Extracts of each tissue sample were prepared by grinding fresh leaves or nodules in a 1:50 (w/v) ratio in PBS-T 20 (neutral 0.01 M phosphate buffer with 0.01% Tween-20). Each sample was assayed in duplicate in two microtiter plates. A purified preparation of SMV was diluted in PBS-T 20 to approximately 10 μg and included as control. Concentrations of SMV coating and conjugated immunoglobins were adjusted to 10 $\mu g \cdot ml^{-1}$, and to approximately 5 $\mu g \cdot ml^{-1}$, respectively, based on optimum detection of SMV-B in preliminary tests. Anti-SMV-IgG was prepared from SMV antiserum (2) by $(NH_4)_2SO_4$ precipitation. Purified SMV and SMV-antiserum were provided by J. H. Hill. The $A_{405\ nm}$ of the leaf and nodule samples was measured and recorded by means of a Titertek Multiskan (Flow Laboratories, Inc., 7655 Old Springhouse Road, McLean, VA 22101).

RESULTS

Reaction of *G. max* and *G. soja* to SMV-B. Symptoms of SMV-B infection of *G. max* Hill and Essex and *G. soja* PI 424-005 and PI 378-693-B, induced in the greenhouse, ranged from severe foliar

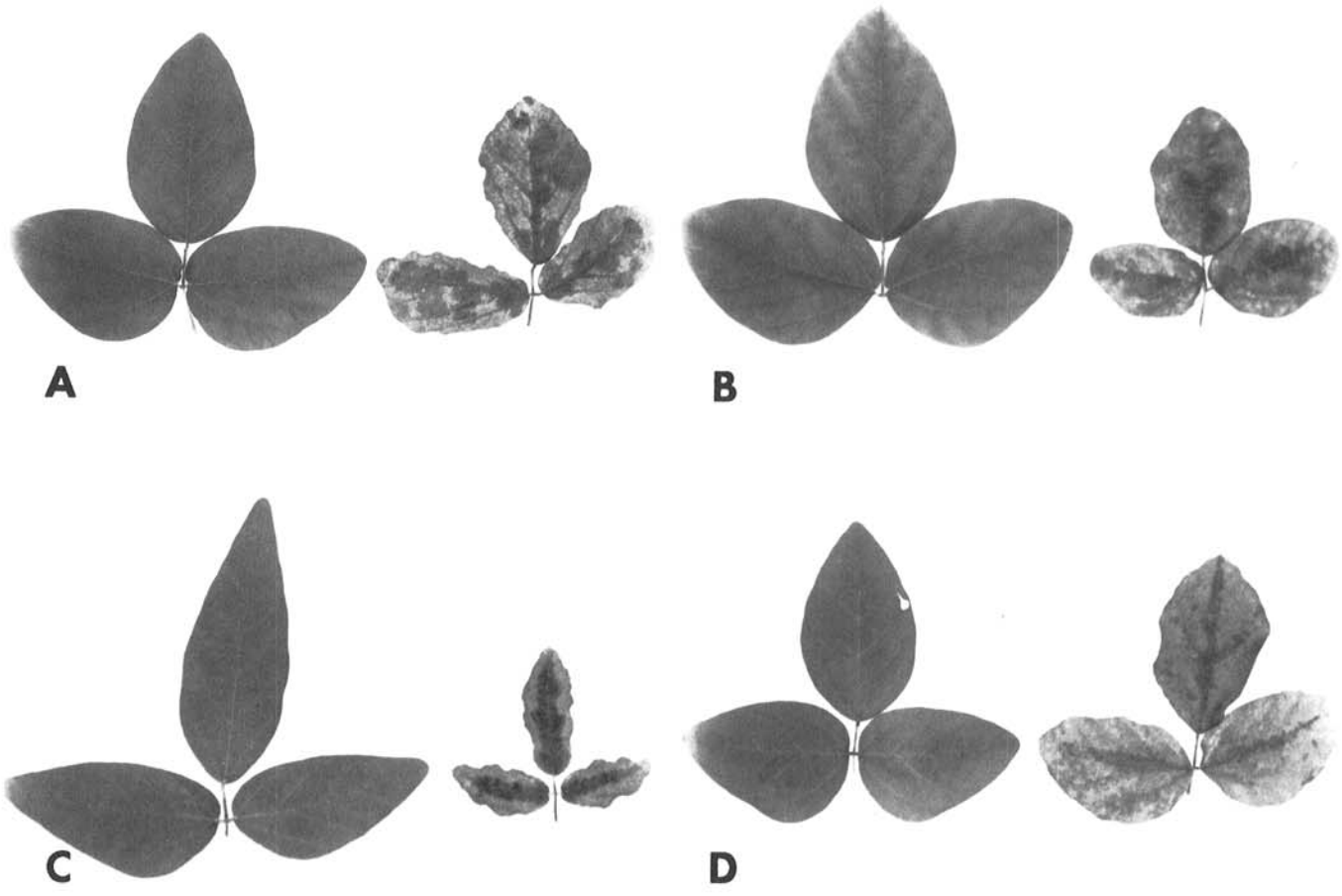

Fig. 1. Mosaic symptoms induced on soybeans by mechanical inoculation with SMV-B (soybean mosaic virus, Beltsville isolate) on **A and B**, *Glycine max* cultivars Hill and Essex, and on **C and D**, *G. soja* PI 424-005 and PI 378-693-B, in the greenhouse. Plants were inoculated while in the primary leaf stage and were photographed 60 days later. At the left of each photo are the healthy controls.

chlorotic mottle and foliar rugosity and severe-to-moderate stunt to transient mottle, rugosity, and slight-to-no stunt (Fig. 1). Symptoms were more severe on PI 424-005 followed with less severe symptoms on Essex, Hill, and PI 378-693-B. The severe symptoms of PI 424-005 and the mild symptoms of PI 378-693-B were consistent in several controlled inoculation experiments. Symptom severity on cultivars Hill and Essex varied from slight to moderate.

Effect of SMV-B on plant growth and nodule mass. SMV-B infection of plants of the four soybean genotypes inoculated in the unifoliate VI stage resulted in significant ($P \leq 0.05$) decreases in mean top weight (grams per dry plant) and corresponding decreases in nodule mass (grams per fresh plant) as shown by weights recorded 53 days after virus inoculation as compared with the uninoculated controls (Table 1). Percent reduction in top weight among genotypes was highest for SMV-B inoculated Essex and lowest for Hill. Decreases in nodule mass, which resulted from virus inoculation, were highest and lowest for PI 378-693-B and PI 424-005, respectively.

Effects of SMV-B on total N, ureide-N, and symbiotic N_2-fixation activity. SMV-B infection of *G. max* Hill and Essex and *G. soja* PI 424-005 and PI 378-693-B resulted in: increases in total N content (milligrams per gram, dry weight basis) in plant tops of all four genotypes, although the increase in Hill was not significant as compared with the virus-free controls (Table 2); the ureide analysis of these infected tissues showed a significant increase in ureide-N content in *G. soja* PI 424-005 and PI 378-693-B as compared with the *G. max* genotypes; and N_2-fixation activity of these genotypes, as indicated by the acetylene reduction assay, decreased significantly in nodules of three of the four genotypes as shown by the percent decreases in activity in *G. max* cultivar Essex, *G. soja* PI 424-005, and PI 378-693-B. Among these soybean genotypes, *G. soja* PI 424-005 had the largest decrease in symbiotic activity (Table 2). In addition, as shown by results obtained by calculating nodule specific activity for nitrogenase as described in Materials and Methods, it was found that whereas nodule specific activity for virus-infected Hill and PI 378-693-B increased 69 and 50%, respectively, nodule specific activity for Essex and PI 424-005 decreased 16 and 88%, respectively (Table 3). The large decrease in specific activity for PI 424-005 nodules indicated that virus infection brought about significant impairment of the symbiotic process in this genotype.

Detection of SMV-B by ELISA. The mean absorbance values obtained by ELISA with leaf extracts of three of the genotypes tested were two to three times greater than those of the corresponding nodule extracts. Differences were greater for PI 378-693-B since absorbance values for nodule extracts were very low (Table 3). ELISA indicated also that SMV-B infected leaves and nodules of cultivar Hill at the time of sampling, had the highest virus concentrations recorded. SMV-B nodules of *G. soja* apparently accumulated very little virus as compared with nodules of *G. max*.

DISCUSSION

The pathological and physiological effects of SMV on soybean of *G. max* and *G. soja* are demonstrated in the present study by consistent reductions in top dry weight, nodule fresh weight, and in N_2-fixation activity per plant. N_2-fixation activity, however, was not significantly reduced in virus-infected plants of *G. max* cultivar Hill. Reduced photosynthesis and photosynthate transport, which are frequently impaired in plants with systemic virus infections (7),

TABLE 1. Effect of SMV-B (soybean mosaic virus, Beltsville isolate) on plant growth and nodule mass of *Glycine max* and *G. soja* at the pod-fill stage

Soybean host[v]	DS[w]	Plant tops (grams dry wt plant^{-1})		Percent change[y]	Nodules (grams fresh wt plant^{-1})		Percent changed[y]
		SMV-B$^-$[x]	SMV-B$^+$		SMV-B$^-$	SMV-B$^+$	
G. max							
Hill (V)	2	2.98 a[z]	2.29 b	−23	0.19 c	0.07 cd	−61
Essex (V)	3	2.56 ab	1.28 cd	−50	0.38 b	0.17 cd	−53
G. soja							
PI 424-005 (IV)	4	1.68 c	0.87 d	−48	0.10 cd	0.07 d	−35
PI 378-693-B (VII)	1	2.88 a	1.60 c	−44	0.55 a	0.12 cd	−77

[v] Plants grown from seed treated with *Rhizobium japonicum* (USDA strain IIb-110). Roman numerals in parentheses indicate maturity group.
[w] Based on visual symptoms according to a disease severity scale in which 0 indicates no symptoms and 5, all plants killed.
[x] The − and + signs next to SMV-B indicate that plants were uninoculated or inoculated, respectively.
[y] Percent change (reduction or increase) expressed as: $\{[(\text{SMV-B}^-)-(\text{SMV}-\text{B}^+)]/(\text{SMV}-\text{B}^-)\} \times 100$.
[z] Mean values in both − and + columns for each growth parameter followed by a different letter are statistically significant ($P \leq 0.05$) according to Duncan's new multiple range test (12).

TABLE 2. Effect of SMV-B (soybean mosaic virus, Beltsville isolate) on total N, ureide-N, and N_2-fixation activity of *Glycine max* and *G. soja* at the pod-fill stage

Soybean cultivars and lines[t]	Total N[u] (dry plant tops in mg·g^{-1})		Percent change[y]	Ureide-N[v] (dry plant tops in mg·g^{-1})		Percent change[y]	N_2-fixation activity[w] (μmoles C_2H_4·plant^{-1}·hr^{-1})		Percent change[y]
	SMV-B$^-$[x]	SMV-B$^+$		SMV-B$^-$	SMV-B$^+$		SMV-V$^-$	SMV-B$^+$	
G. max									
Hill	23.1 d[z]	25.7 d	+11	0.61 bc	0.60 bc	− 2	3.37 b	2.10 b	−38
Essex	25.4 d	33.8 b	+33	0.69 bc	0.76 bc	+ 10	4.98 a	1.88 b	−62
G. soja									
PI 424-005	29.7 c	40.4 a	+36	0.46 c	1.99 a	+333	2.31 b	0.20 c	−91
PI 378-693-B	23.4 d	33.6 b	+43	0.69 bc	0.90 b	+ 38	6.12 a	2.01 b	−67

[t] Plants grown from seed treated with *Rhizobium japonicum* (USDA strain IIb-110). For maturity group and disease severity see footnotes v and w in Table 1.
[u] Determined by the Kjeldahl method.
[v] For analytical method, see the Materials and Methods section.
[w] Expressed by the rate of acetylene (C_2H_2) reduction to ethylene (C_2H_4).
[x] The − and + signs indicate that plants were uninoculated or inoculated, respectively.
[y] Percent change (reduction or increase) expressed as: $\{[(\text{SMV-B}^-)-(\text{SMV}-\text{B}^+)]/(\text{SMV}-\text{B}^-)\} \times 100$.
[z] Mean values in both − and + columns for each functional parameter followed by a different letter are statistically significant at the $P \leq 0.05$ level according to Duncan's new multiple range test (12).

can be implicated in the decreases in nodule weight and N_2-fixation activity as opposed to concluding that virus replication in the nodule interfered directly with the energy-dependent *Rhizobium*-host symbiosis. The indirect effect of virus replication seemed to be particularly evident in *G. max* cultivar Hill and in *G. soja* PI 378-693-B, where nodule specific activity for SMV-B infected plants was significantly increased as compared to that for the virus-free controls. This enhanced nodule response might result from an infected plant's ability to compensate for virus-induced physiological stress by increasing the N_2-fixing capacity of the symbiotic system as has been demonstrated for nodulated legumes under environmental stress (8). Nodule specific activity for virus-infected plants of *G. soja* PI 424-005 was severely reduced and may indicate a very different virus-host-*Rhizobium* interaction.

In contrast to SMV-B-induced decreases in soybean plant weights and plant N_2-fixation activity, the virus induced significant increases in total N in *G. max* and *G. soja*, with exception of *G. max* cultivar Hill for which a slight increase was recorded. Total N increases in virus-infected plants may be attributed to their reduced growth and pod-set (*unpublished*), which probably restricted the assimilation of the nitrogen available to the plant through the fixation process. Ureides (allantoin and allantoic acid), which are products of purine metabolism and transport molecules of fixed nitrogen, were also increased in virus-infected plants of *G. soja*, whereas in *G. max*, the differences between the virus and the no-virus treatments were not significant. The disruptive effects of SMV-B on plant metabolism were particularly evident in the *G. soja* PI 424-005 genotype where a large increase in ureide-N occurred simultaneously with low nodule specific activity for nitrogenase. In healthy nodulated soybeans it has been reported that ureide-N concentrations, particularly in xylem sap, are closely associated with N_2-fixation activity (13). Ureide-N accumulation in SMV-B infected PI 424-005 plants may have resulted from virus-enhanced purine catabolism in the leaves or impaired ureide utilization.

The severity of mosaic symptoms of plants of both soybean species under greenhouse conditions could not be consistently associated with specific plant reaction to the virus (Tables 1 and 2) or with virus titer as determined by ELISA (Table 4). *G. max* cultivar Hill, which apparently had the highest virus concentration in leaves and nodules, exhibited the mildest disease symptoms in response to inoculation with the SMV-B isolate. Conclusions based on ELISA must be made with caution since the tissues assayed represented composite samples from a single sampling date (15). The apparent tolerant reaction of *G. max* cultivar Hill to SMV-B merits further evaluation to determine its yield response to this or to other SMV isolates under field conditions.

The effects of SMV on symbiotic N_2-fixation and ureide-N accumulation in soybean exhibiting differential responses to the disease could be further clarified by determining the virus effects on ureide transport in xylem sap during the growth cycle of nodulated and non-nodulated soybeans. Further research is also needed in screening wild soybean germplasm for resistance to SMV.

TABLE 3. Effect on SMV-B (soybean mosaic virus, Beltsville isolate) on nodule specific activity for nitrogenase in *Glycine max* and *G. soja* genotypes

Soybean host[v]	Nodule specific activity[w,x]		Percent change[y]
	SMV-B$^-$	SMV-B$^+$	
G. max			
Hill	15.54 bc[z]	30.00 a	+69
Essex	13.63 bc	11.05 cd	−16
G. soja			
PI 424-005	21.45 b	2.85 d	−88
PI 378-693-B	11.24 c	16.05 bc	+50

[v] Plants grown from seed treated with *Rhizobium japonicum* (USDA strain (I1b-110). For maturity group and disease severity see footnotes v and w in Table 1.

[w] Activity for nitrogenase expressed as (μmoles C_2H_4 plant^{-1}·hr^{-1})/(g of nodules·plant^{-1}).

[x] The − and + signs indicate that the plants were uninoculated or inoculated with SMV-B, respectively.

[y] Reduction or increase expressed as $\{[(\text{SMV-B}^-)-(\text{SMV-B}^+)]/(\text{SMV}-\text{B}^-)\} \times 100$.

[z] Mean values in − and + columns for nodule specific activity followed by a different letter are statistically significant ($P \leq 0.05$) according to Duncan's new multiple range test (12).

TABLE 4. Detection of SMV-B$^+$ (soybean mosaic virus, Beltsville isolate) in *Glycine max* and *G. soja* genotypes by means of the enzyme-linked immunosorbent assay (ELISA)

Soybean host[a]	Absorbance (405 nm)	
	Leaves[b]	Nodules[b]
G. max		
Hill	0.932[c] ± 0.13	0.304 ± 0.02
Essex	0.619 ± 0.09	0.245 ± 0.03
G. soja		
PI 424-005	0.361 ± 0.07	0.102 ± 0.04
PI 378-693-B	0.780 ± 0.06	undetectable

[a] Plants inoculated with SMV-B in the unifoliate VI stage.

[b] For sampling method see the Materials and Methods section.

[c] Means and SEs of samples replicated twice in two microplates. Values corrected for absorbance of the virus-free controls.

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