

Free Amino Acids in Soybeans Infected with Soybean Mosaic Virus, Bean Pod Mottle Virus, or Both

J. C. Tu and R. E. Ford

Postdoctoral Research Associate and Professor, respectively, Department of Botany and Plant Pathology, Iowa State University, Ames 50010. Present address of senior author, Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada.

Journal Paper No. J-6370 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project No. 1628.

The authors thank S. S. Quiniones and J. M. Dunleavy for the viruses and Richard Ewan for assistance with biochemical analysis.

Accepted for publication 4 November 1969.

ABSTRACT

Free amino acids were measured quantitatively in soybean (*Glycine max*) leaves infected with three soybean mosaic virus (SMV) isolates differing in virulence and with bean pod mottle virus (BPMV) alone or in combination with SMV. Total free amino acids and ammonia increased with virus infection. Individual amino acids decreased, however, or were variable depending on the SMV isolate. Free amino acid content appeared closely correlated

with symptom severity. Free amino acid concentration increases were in order of SMV-O- > SMV-NC- > SMV-M-infected soybean.

BPMV and SMV-O had a synergistic effect on soybeans in some free amino acids, because free amino acid increased in mixed infections but did not increase in proportion with increased disease severity. *Phytopathology* 60:660-664.

The relationship of symptom severity with free amino acid changes in virus-infected plants is not clear (5). The uncertainty is based on whether free amino acid concentration increase in an infected plant is a result, or the cause, of the disease symptoms.

Virus diseaselike symptoms have been induced by supplying excess concentrations of certain amino acids to a healthy plant (5, 13). Commoner & Nehari (3) suggested that virus disease symptoms might result from free amino acid and amide increase or decrease in infected plants. Free amino acid increase and symptomatology, however, were not correlated in infections of potato viruses X or Y (1).

In maize dwarf mosaic virus- (MDMV) infected corn, a correlation of symptom severity and free amino acid increases was found at the strain level. The correlation was clear only in newly emerged systemically infected leaves (7). No mosaic symptoms resulted when leaves were inoculated with MDMV after maturation, even though similar free amino-acid concentration increase and virus multiplication occurred (Tu & Ford, unpublished data). The phenomenon suggests that mosaic symptoms of MDMV resulted from underdevelopment of chloroplasts or insufficient chlorophyll synthesis during the processes of cell division and maturation in some virus-infected cells in the leaf primordium. Thus, the symptom severity of a virus-infected plant reflects the degree of metabolic disturbance resulting from virus multiplication or abnormal host metabolites.

The symptom severity of several virus isolates or strains in a host varies, indicating differences in virulence among the isolates (12). Often, closely related isolates manifested similar physiological changes in a host. For example, Pa. 1 (8) and Ia. 188 (6) isolates of MDMV-B are more closely related to sugarcane mosaic virus (SCMV) strain H than to MDMV-A (2), and they manifested similar free amino acid changes in infected corn (7).

The purposes of this study are to (i) determine free amino acid concentration changes induced by soybean mosaic virus (SMV) isolates of differing virulence; (ii) correlate virulence with symptom severity and virus multiplication in newly formed systemic leaves; and (iii) correlate symptom severity with virus multiplication and free amino acid concentrations in mixed infections of SMV and bean pod mottle virus (BPMV) in soybean.

The correlation among symptom severity, virus multiplication, and free amino acid concentration changes was investigated by using several SMV isolates that caused different symptom severity on *Glycine max* (L.) Merr. 'Bansei' soybean (9) rather than on different virus-host combinations.

BPMV alone causes a leaf mottle, and SMV alone causes a leaf mosaic and mild rugosity in infected Bansei soybean. Bansei soybean infected with a SMV and BPMV mixture causes a severe rugosity of leaves, dwarfing, delayed flowering and maturation, and greater yield reductions than soybeans infected with either virus alone, suggesting a synergistic effect (10, 11).

MATERIALS AND METHODS.—SMV isolates used in this study were SMV-NC, SMV-M, and SMV-O (9). SMV-NC was obtained from J. P. Ross, North Carolina; SMV-M was isolated from Hood soybean (*Glycine max* 'Hood') at Ames, Iowa; and SMV-O and BPMV were isolated from soybeans collected at Ottumwa, Iowa. Mixtures of these viruses and BPMV alone were also used. Bansei soybean was used because it expressed clearly different symptom severities to the several SMV isolates.

Five soybean seeds were sown in each of 360 6-inch clay pots containing steamed soil. The pots were divided into four groups. The leaves of the control group were dusted with 600-mesh Carborundum and rubbed with phosphate buffer to simulate inoculation. The other three groups were Carborundum-dusted and

inoculated with SMV-NC, SMV-M, or the mixture (SMV-O + BPMV), respectively. A second experiment, June 1968, consisted of an additional two groups of 90 pots each in addition to the four groups already mentioned. One of these additional groups was inoculated with SMV-O, and the other was inoculated with BPMV. Both experiments were identically planted, inoculated, and harvested.

Inocula were crude sap obtained from grinding 2-week-old SMV-infected soybean leaves with equal amounts (w/v) of 0.01 M neutral phosphate buffer. The ground tissues were strained through two layers of cheesecloth. The sap was diluted 1:10 with the same buffer. Inoculations were made at the unifoliolate stage by rubbing inocula on plants predusted with 600-mesh Carborundum with a pestle. After inoculation, the pots were kept in a 24-C greenhouse and watered with tap water. Infectivity assays for determination of virus titer were made on Bansei soybean. Assays were dilution end point (DEP) determinations, since reliable local lesion hosts for SMV were not available. Although local lesion hosts for BPMV are known, we used a similar assay (DEP) for it in the interest of uniformity and to aid final interpretation and comparison of infectivity results.

Leaves were harvested for amino acid analysis at the fourth-trifoliolate stage. About 4 weeks after inoculation, leaves above the second trifoliolate were collected, placed in plastic bags, and frozen immediately at -20 C.

Before harvest, some fully expanded fourth-trifoliolate leaves were collected from each treatment. Crude sap from leaves of each treatment was assayed for dilution end point. Samples for free amino acid analysis were prepared as before (7). Slight modifications in processing were made to accommodate soybeans. First, the crude sap was diluted 1:3 with distilled water before an equal amount of ether was introduced, because the crude sap of soybean contained considerable amounts of lipids and related compounds that gelatinized upon addition of ether. Second, low-speed (10,400 g) centrifugation was applied to break the interphase of gelatinous network and to allow the trapped solution to partition into the aqueous phase for better recovery. Third, proteins precipitated by picric acid were satisfactorily removed at 16,300 g for 30 min instead of at high-speed centrifugation. These modifications provided

a good recovery of free amino acid after each treatment.

Fifty ml each of crude sap derived from leaves were processed. Finally, each sample was evaporated to dryness in a flash rotary evaporator at 50 C. The dried material was resuspended in 50 ml of pH 2.2 sodium citrate buffer. One ml was applied to each column of a Technicon (Type A chromobeads) automatic amino acid analyzer for separation. Identifications of amino acids were made by comparing the relative position of peaks recorded in chromatographic charts from analytic samples with those of a known synthetic amino acids mixture. The molarity of each amino acid was converted directly from the area of each peak in the chromatographic charts.

RESULTS.—Infectivity vs. symptom severity.—The DEP of 10^{-4} for SMV and 10^{-5} for BPMV (Table 1) agrees with those reported by Quiniones (9). No difference in DEP was found for several SMV isolates in crude sap of infected Bansei soybean. Among the three isolates, however, slightly higher infectivity was noted for SMV-O than for SMV-NC or SMV-M. Despite the same DEPs for different SMV isolates, observed symptom severity was related to the relative infectivity of different isolates (Table 1).

BPMV had a DEP of 10^{-5} . DEP for BPMV plus SMV-O was 10^{-5} , but the infected assay-plants showed symptoms of mixed infection up to 10^{-4} , and, at 2×10^{-5} , on the assay plants, only BPMV-disease symptoms appeared. Evidently, a BPMV and SMV-O mixed infection had no effect on the DEP of either virus.

Even though the DEP for BPMV or SMV-O did not change in the BPMV-SMV-O-infected Bansei soybeans, symptom severity was greatly increased. Thus, symptom severity in this instance was not directly correlated with virus titer.

Healthy vs. SMV-diseased leaves.—Discounting the free amino acid concentration differences between different SMV isolates, free amino acid concentration increased in all SMV infected leaves. The increase ranged from approximately 28 to 106%, depending on the virulence of isolates (Table 2). SMV-O, the most virulent isolate, induced a 104% concentration increase of free amino acids in infected leaves. SMV-NC, an intermediate, and SMV-M, the least virulent isolate, caused 83 and 28% increases in free amino acid concentrations in infected Bansei.

Asparagine, aspartic acid, serine, and alanine in-

TABLE 1. Dilution end point assay for crude sap from various soybean mosaic virus (SMV) isolates and/or bean pod mottle virus (BPMV) infected Bansei soybean. Assays were made from freshly sampled leaves immediately before the leaves were harvested for free amino acid analysis

| Virus | Dilution | | | | | | | | | Symptom severity |
|--------------|--------------------|-----------|-----------|-----------|--------------------|-----------|--------------------|-------------------|-----------|------------------|
| | 10^0 | 10^{-1} | 10^{-2} | 10^{-3} | 2×10^{-4} | 10^{-4} | 2×10^{-5} | 10^{-5} | 10^{-6} | |
| SMV-O | 19/20 ^a | 19/20 | 17/19 | 7/20 | 4/19 | 3/18 | 0/20 | 0/18 | 0/20 | +++ |
| SMV-NC | 19/19 | 20/21 | 15/20 | 5/19 | 2/20 | 1/20 | 0/20 | 0/20 | 0/20 | ++ |
| SMV-M | 18/20 | 19/20 | 15/21 | 3/20 | 2/20 | 1/19 | 0/19 | 0/20 | 0/20 | + |
| BPMV | 19/20 | 18/20 | 14/19 | 11/20 | 4/20 | 3/20 | 3/18 | 1/20 | 0/19 | + |
| BPMV + SMV-O | 19/20 | 19/19 | 17/20 | 14/20 | 6/19 | 5/20 | 3/19 ^b | 2/20 ^b | 0/20 | ++++ |

^a Figures are total of two replicate tests; numerator = plants infected; denominator = plants inoculated.

^b Infections (enumerator) showed only BPMV-diseased symptom, but not symptoms of BPMV-SMV mixture.

creased in leaves infected with different SMV isolates, and systemic mosaic symptoms were correlated with free amino acid concentration increase (Table 2).

Many free amino acids not often detectable in healthy soybean leaves were detected in diseased leaves. In healthy soybean leaves, 15 of the 20 amino acids examined were detectable. Citrulline, cysteine, phenylalanine, histidine, and arginine were not found in healthy leaves, whereas 16, 19, and 20 amino acids were detected in leaves infected with SMV-M, SMV-NC, and SMV-O, respectively. Citrulline, cysteine, histidine, and arginine were not detected in SMV-M infected leaves. Only cysteine was not detectable in SMV-NC infection. The number of detectable free amino acids increased seemingly with increasing disease severity in infected Bansei soybean.

In SMV-NC- and SMV-O-infected leaves, all detectable free amino acids increased in concentration when compared with healthy Bansei soybean, and some decrease occurred in SMV-M infected leaves (Table 2).

Mixed vs. SMV-O or BPMV infection alone.—It was shown earlier that total free amino acid concentration increased approximately 106% from healthy in SMV-O infected leaves. Total free amino acid concentration in leaves infected with BPMV increased about 69% when compared with uninfected plants. Increase in total free amino acid concentration in the mixture-infected leaves was 73% of the comparable healthy control (Table 2). Disease severity was in the descending order, mixture (BPMV + SMV-O), SMV-O, and BPMV-infected Bansei soybean. Correlation between symptom severity

and free amino acid concentration increase did not exist.

The total free amino acid concentration in the mixture-infected leaves did not have a marked increase, but remained approximately the same as that of BPMV infection. However, shifts in concentration in some individual amino acids occurred.

Comparisons between mixture and BPMV infection indicated that the mixture had 14 amino acids lower, 3 unchanged, and 3 higher than BPMV alone. The three increasing were aspartic acid, asparagine, and glutamic acid. When a similar comparison was made between SMV-O and the mixture, all amino acids in the mixture-infected leaves were lower except aspartic acid, which increased considerably.

Five free amino acids were not detected in healthy leaves, but all five were detectable in SMV-O infected leaves. In BPMV-infected leaves, only arginine was not detectable. In the mixture-infected leaves, arginine, cysteine, and citrulline were not detectable.

DISCUSSION.—If sap dilutions from infected leaves were assayed for DEP or infectivity on the same host cultivar at the same growth stage under identical conditions, either or both could reflect the relative virus concentration in the saps. DEP assay, however, is not the most sensitive test for infectivity (4). Our results showed that Bansei soybean infected with three different isolates had the same DEP but had slightly different infectivities. The infectivity differences were more obvious in dilutions between 10^{-2} and 2×10^{-4} (Table 1). Possibly at a given dilution, the one with

TABLE 2. Quantity of free amino acids and ammonia in 50 ml of leaf sap from healthy and soybeans infected with soybean mosaic virus isolate O (SMV-O), isolate M (SMV-M) and isolate NC (SMV-NC), and bean pod mottle virus (BPMV) or a mixture of the two viruses (SMV-O + BPMV)

| | Healthy ^a | SMV-M ^a | SMV-NC ^a | SMV-O ^b | BPMV ^b | SMV-O ^b + BPMV |
|---------------|----------------------|--------------------|---------------------|--------------------|-------------------|------------------------------|
| | μmoles | μmoles | μmoles | μmoles | μmoles | μmoles |
| Alanine | 45.6 | 50.6+ ^c | 65.8+ | 84.7+ | 70.4+ | 62.4+ |
| Arginine | N.D. ^d | N.D. | 5.0+ | 2.4+ | N.D. | N.D. |
| Asparagine | 234.9 | 321.3+ | 442.8+ | 489.6+ | 456.9+ | 484.6+ |
| Aspartic acid | 24.4 | 51.5+ | 65.2+ | 54.0+ | 52.2+ | 73.9+ |
| Citrulline | N.D. | N.D. | 1.7 | 2.0 | 0.3 | N.D. |
| Cysteine | N.D. | N.D. | N.D. | 0.9 | 0.7 | N.D. |
| Glutamic acid | 17.7 | 18.8 | 18.1 | 18.7 | 15.2 | 17.0 |
| Glycine | 14.6 | 12.5- | 22.7+ | 33.4+ | 17.0+ | 13.2- |
| Histidine | N.D. | N.D. | 2.8+ | 2.4+ | 1.8+ | 0.8+ |
| Isoleucine | 11.1 | 14.0+ | 21.1+ | 23.7+ | 12.7+ | 12.6+ |
| Leucine | 15.1 | 13.8- | 20.7+ | 26.1+ | 14.3 | 13.1- |
| Lysine | 5.1 | 5.4 | 14.0+ | 14.4+ | 11.9+ | 6.0+ |
| Methionine | 2.3 | 2.0 | 3.7+ | 4.5+ | 2.4 | 2.0 |
| Ornithine | 2.5 | 1.7- | 6.5+ | 7.4+ | 2.9 | 1.1- |
| Phenylalanine | N.D. | 5.9+ | 21.4+ | 12.8+ | 7.7+ | 2.6+ |
| Proline | 14.3 | 18.7+ | 16.8+ | 21.0+ | 18.2+ | 13.2- |
| Serine | 65.0 | 75.1+ | 114.3+ | 145.5+ | 98.7+ | 92.7+ |
| Threonine | 19.3 | 19.2 | 27.2+ | 30.9+ | 22.0 | 22.0 |
| Tyrosine | 3.1 | 1.7- | 5.1+ | 6.3+ | 3.9+ | 2.0- |
| Valine | 11.9 | 12.9+ | 14.4+ | 21.8+ | 14.5+ | 13.9+ |
| Total | 486.9 | 625.1 | 889.3 | 1002.5 | 823.7 | 833.1 |

^a An average of four runs; two from samples harvested in September 1967, two from samples harvested in May 1968. All analyses were done in the Technicon automatic analyzer.

^b An average of two runs from sample harvest in May 1968.

^c + = A significant increase compared to healthy; - = a significant decrease at the 5% level.

^d N.D. = None detected.

higher infectivity contains more virus particles than the others. Symptom severity was positively correlated with infectivity for different isolates.

Similarly, when infectivities of BPMV and SMV-O alone were compared with that of BPMV + SMV-O, only a slight increase in infectivity was noted in the virus mixture, but no increase in DEP was found. Thus, the increase in symptom severity did not result merely from virus-titer increase, but was probably caused by a greater physiological disturbance due to the interaction of the two unrelated viruses.

The virus infectivity increase in the mixture infection (SMV-O + BPMV) and the infectivity difference among the SMV isolates may be interpreted separately on the basis of our data (Table 2).

Infectivity difference among the SMV isolates.—According to Liebig's law of minimum and limiting factor theory, one or more of the amino acids listed in Table 2 may be a limiting factor determining the amount of total viral protein production, possibly one which is lower in concentration. Different virus strains or isolates could differ in one or two amino acids in their peptides, or even more in more distantly related virus strains (14). Thus, the proportion of amino acids required for viral protein synthesis differed in different strains or isolates.

It is reasonable to assume that a healthy plant at a given time has a definite composition of free amino acids in the free amino acid pool. Infection by different isolates withdraws different proportions of free amino acid necessary for viral protein synthesis. If an isolate used more amino acid, which is a limiting factor, per unit of viral protein, the isolate would have fewer particles synthesized when compared with an isolate that used less of the limiting free amino acid. For example, if free amino acid A is a limiting factor and has a concentration X_{UM} , and if isolates I and II, respectively, need 10 and 5 amino acid A/unit of protein synthesized, and the concentration of final protein is K_1 for isolate I and K_2 for isolate II, the formula would

be written as: $K_1 = \frac{X}{10}$; $K_2 = \frac{X}{5}$. $\therefore K_1 = \frac{1}{2}K_2$. Since

the isolate used less limiting amino acid and gave more virus protein synthesis, it would also use more other nonlimiting amino acids.

The decrease of free amino acid critical to virus protein synthesis perhaps could in turn stimulate total amino acid synthesis in general. The result of this sequence of events might result in an accumulation of free amino acids.

Infectivity decrease in different SMV isolates was inversely correlated with a number of free amino acids not detectable. It is reasonable to suspect that one or more of these undetectable free amino acids are, possibly, limiting factors. If a limiting amino acid is applicable for both host and viral protein synthesis, competition for the limiting amino acid occurs. Less viral protein synthesis may result. Furthermore, if more than one limiting factor is involved, the chance for greater

limitation of virus multiplication increases. This phenomenon appeared applicable to our data (Table 2).

Infectivity increase in mixture infection.—No arginine was detected in healthy and BPMV-infected soybean, but arginine was available in SMV-O-infected leaves (Table 2). Therefore, arginine may be a limiting free amino acid for both host-protein and BPMV-protein synthesis. Even though arginine synthesis may have been induced after virus infection, the increased production still could not fulfill the need for BPMV and host. On the other hand, a slight accumulation of arginine in SMV-O-infected leaves could be interpreted as (i) that the virus-stimulated arginine synthesis had produced more arginine than host and SMV-O's needs; or (ii) that host-protein synthesis slowed down after viral infection, some unused arginine started to accumulate, and arginine was not a limiting factor for SMV-O.

Nevertheless, in the mixture-infected plants, the arginine demanded by BPMV may be compensated by SMV-O infection (Table 2). Thus, BPMV synthesis may continue until the exhaustion of arginine or until some other amino acid is in short supply. No excess free amino acid accumulated, even though a greater amount of virus synthesis for BPMV and SMV-O in the mixed infection occurred. The total free amino acid accumulation in the mixture-infected leaves was 833 μ moles, which fell between the total free amino acid concentration in SMV-O or BPMV-infected leaves (Table 2).

Symptom severity could be correlated with virus multiplication rates and/or free amino acid accumulation only when the comparisons were made within infections with strains or isolates. Correlation of symptom severity with virus multiplication for different viruses may be incidental. Symptom severity in a mixture infection caused by two different virus isolates appeared more complicated than simply the virus multiplication and free amino acid accumulation.

LITERATURE CITED

1. BOZARTH, R. F., & T. O. DIENER. 1963. Changes in concentration of free amino acids and amides induced in tobacco plants by potato virus X and potato virus Y. *Virology* 21:188-193.
2. BUCHHOLTZ, W. F., R. E. FORD, & R. C. LAMBE. 1966. Maize dwarf mosaic virus in Iowa. *Plant Dis. Reprtr.* 50:88.
3. COMMONER, B., & V. NEHARI. 1953. The effects of tobacco mosaic virus synthesis on the free amino acid and amide composition of the host. *J. General Physiol.* 35:791-805.
4. CORBETT, M. K., & H. D. SISLER. 1964. *Plant Virology*. Univ. Fla. Press, Gainesville. 527 p.
5. DIENER, T. O. 1963. Physiology of virus-infected plants. *Annu. Rev. Phytopath.* 1:197-218.
6. FORD, R. E., W. F. BUCHHOLTZ, & R. C. LAMBE. 1967. Occurrence of maize dwarf mosaic virus in Iowa in 1966. *Plant Dis. Reprtr.* 51:388-389.
7. FORD, R. E., & J. C. TU. 1969. Free amino acid contents in corn infected with maize dwarf mosaic virus and sugarcane mosaic virus. *Phytopathology* 59:179-182.
8. MACKENZIE, D. R., C. C. WERNHAM, & R. E. FORD. 1966. Differences in maize dwarf mosaic virus isolates

- of northeastern United States. Plant Dis. Repr. 50: 814-818.
9. QUINIONES, S. S. 1968. Soybean mosaic. Ph.D. Thesis. Iowa State Univ., Ames. 74 p.
 10. ROSS, J. P. 1965. Effect of infection sequence of bean pod mottle and soybean mosaic viruses on host reaction and virus titers in doubly infected soybean shoot apices. Phytopathology 55:1074 (Abstr.).
 11. ROSS, J. P. 1968. Effect of single and double infections of soybean mosaic and bean pod mottle viruses on soybean yield and seed characters. Plant Dis. Repr. 52:344-348.
 12. ROSS, J. P. 1969. Pathogenic variation among isolates of soybean mosaic virus. Phytopathology 59:829-832.
 13. WARIS, H. 1962. Neomorphosis in seed plants induced by amino acids: II. *Oenanthelachenalii*. Physiology Plantarum 15:736-752.
 14. WHITTMANN, H. G. 1960. Comparison of the tryptic peptides of chemically induced and spontaneous mutants of tobacco mosaic virus. Virology 12:609-612.