Natural Infection of Tropical Forage Legume Species of *Arachis* and *Stylosanthes* by Potyviruses Related to Peanut Mottle Virus

F. J. MORALES, Virologist, M. CASTAÑO, Research Associate, A. C. VELASCO, Research Associate, and J. ARROYAVE, Electron Microscopist, Centro Internacional de Agricultura Tropical (CIAT), Apartado Aereo 6713, Cali, Colombia; and F. W. ZETTLER, Professor, Department of Plant Pathology, University of Florida, Gainesville 32611

ABSTRACT

Morales, F. J., Castaño, M., Velasco, A. C., Arroyave, J., and Zettler, F. W. 1991. Natural infection of tropical forage legume species of *Arachis* and *Stylosanthes* by potyviruses related to peanut mottle virus. Plant Dis. 75:1090-1093.

The South American forage legume *Arachis pintoi* was found to be affected by foliar ring spot symptoms in surveys of various localities in the Cauca Valley of Colombia. A filamentous virus (approximately 750 nm) isolated from symptomatic *A. pintoi* plants induced the characteristic ring spot symptoms in manually inoculated virus-free *A. pintoi* plants. This virus also caused systemic infection of other legumes, such as common bean, cowpea, peanut, and soybean, in manual transmission tests. A similar virus was also found in these surveys causing mild chlorosis and leaf malformation of another neotropical forage legume, *Stylosanthes* sp., which is closely related to the genus *Arachis*. The *Stylosanthes* virus systemically infected *Stylosanthes* spp., common bean, cowpea, and peanut by manual inoculation but not soybean. Of the nonlegumes tested, only *Nicotiana benthamiana* was systemically infected by both viruses. The *A. pintoi* and *Stylosanthes* sp. viruses were serologically related to each other and to peanut mottle virus (PMoV), and both induced cytoplasmic inclusions similar to those reported for PMoV. Differences between the viruses isolated in this investigation and PMoV must be studied further before these tropical forage legume potyviruses can be considered strains of PMoV.

The perennial peanut *Arachis pintoi* Krapovickas & Gregory and various species of *Stylosanthes* are highly promising forage legumes used in association with grasses to improve the nutritional quality of pastures in tropical and subtropical regions of the world (8,17).

Despite its South American origin, *A. pintoi* was apparently introduced in Colombia from the United States in a group of 36 accessions of wild *Arachis* species maintained by the University of Florida and the USDA Plant Introduction Center in Georgia (8). In Colombia, *A. pintoi* exhibited excellent adaptation to the infertile, acid soils of important cattle-raising regions in the eastern plains (200 miles above sea level), including resistance to pests and diseases (8). *A. pintoi* was subsequently introduced in the Cauca Valley (1,000 miles above sea level) of Colombia, where it was planted in different localities as a forage crop. A survey of two of these localities in the municipalities of Ginebra and Florida (Valle), however, revealed a high proportion of *A. pintoi* plants affected by foliar ring spot symptoms.

Accepted for publication 30 April 1991 (submitted for electronic processing).

© 1991 The American Phytopathological Society

The neotropical genus *Stylosanthes* is very closely related to the genus *Arachis*, both belonging to the same tribe (Aeschynomeneae) and subfamily (Papilionoideae) of the Leguminosae (17). The CIAT collection of *Stylosanthes* spp. was initiated in 1972 with germ plasm collected in the lowlands of Colombia and other Latin American countries, mainly Brazil (16). Although no viral problems had been observed previously in this genus in the South American continent (11), field surveys conducted during this investigation revealed the occurrence of foliar chlorosis and malformation in some *Stylosanthes* sp. plants grown in the locality of Santander de Quilichao, Cauca, Colombia.

The purpose of this investigation was to characterize the causal agents of the above-mentioned symptoms in *A. pintoi* and *Stylosanthes* sp.

MATERIALS AND METHODS

Virus isolation and maintenance. Stolons of *A. pintoi* plants affected by ring spot symptoms (Fig. 1A) were collected in the municipality of Ginebra (Valle) for vegetative propagation and maintenance. The causal agent was also maintained by manual inoculation in pea (*Pisum sativum* L. 'Alaska'). Leaf samples of *Stylosanthes* sp. plants showing mild chlorosis and leaf malformation (Fig. 1B) were collected in Santander de Quilichao (Cauca) as a source of inoculum. The causal agents of the *A. pintoi* ring spot and *Stylosanthes* sp. chlorosis were maintained in common bean (*Phaseolus vulgaris* L. 'Bountiful') under glasshouse conditions (maximum light intensity of 87 $\mu E \cdot m^{-2} \cdot s^{-1}$, 26 C, and 75% RH, average annual values) by manual inoculation of test plants with infected leaf extracts prepared in 0.1 M KPO_4 buffer, pH 7.0, (1:10, w/v).

Electron microscopy. Leaf extracts and purified preparations from infected test plants were negatively stained in 2% uranyl acetate, pH 3.7, to be examined for the presence of virus particles using a JEOL SX-100 electron microscope. Leaf tissue of symptomatic *A. pintoi* and *Stylosanthes* sp. plants was prepared for cytology as described earlier (13). Thin sections were cut with a diamond knife using a MT 6000 Sorval ultramicrotome.

Pathogenicity tests. Mechanically inoculated legume species included *A. hypogaea* L., *A. pintoi*, *Canavalia* sp., *Senna occidentalis* (L.) H. Irwin & Barneby, *Centrosema brasilianum* (L.) Benth., *Glycine max* (L.) Merr., *P. lunatus* L., *P. vulgaris*, *P. sativum*, *S. capitata* Vog., *S. macrocephala* Ferr. & Costa, *Vigna radiata* (L.) R. Wilcz., and *V. unguiculata* subsp. *unguiculata* (L.) Walp. Other plant species inoculated were *Chenopodium quinoa* Willd., *C. amaranticolor* Coste & Reyn., *Datura stramonium* L., *Gomphrena globosa* L., *Nicotiana benthamiana* Domin., *N. glutinosa* L., *N. tabacum* L., and *Physalis angulata* L. All test plants were serologically assayed 15 days after inoculation.

Insect transmission tests. Individuals of *Myzus persicae* (Sulzer) reared on pepper plants (*Capsicum* sp.) were tested as vectors. Aphids were starved for 1 hr before being transferred to cowpea (cv. Monteria) plants infected with the *A. pintoi* potyvirus or to bean (cv. Bountiful) plants infected with the *Stylosanthes* sp. virus. After a 10-min acquisition access period, aphids were transferred in groups of five to healthy cowpea (*A. pintoi* virus) or to Bountiful bean seedlings (*Stylosanthes* sp. virus) and al-

lowed a 24-hr inoculation access period. Aphids were also transferred to healthy bean and cowpea plants for 24-hr test feedings as controls (none of these plants became infected in this study). After the inoculation period, aphids were killed with an insecticide.

Seed transmission tests. A total of 3,451 *A. pintoi* and 4,880 *Stylosanthes* spp. seeds produced by CIAT were tested by the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for the possible presence of the viruses detected in this investigation. Also, a group of 280 *A. pintoi* seeds harvested from plants affected with ring spot were tested by ELISA for the presence of the virus isolated from this legume. The seeds were soaked overnight in sterile distilled water and then homogenized in groups of 10 seeds to prepare one test sample.

Virus purification. The *A. pintoi* and *Stylosanthes* sp. viruses were propagated in pea (cv. Alaska) and bean (cv. Dubbele Witte), respectively. These viruses were purified by homogenizing infected tissue in a blender with 2 ml of cold 0.5 M KPO_4 buffer, pH 7.6, containing 0.5 g of Na_2SO_3 and 1 mM of Na-DIECA per gram of infected tissue. The resulting mixture was further homogenized in the presence of chloroform and carbon tetrachloride, using 0.5 ml of each solvent per gram of plant tissue. After centrifugation at 4,080 *g* for 5 min, the supernatant was treated with 5% PEG and stirred for 1 hr at 5 C. The virus was precipitated by centrifugation at 8,000 *g* for 20 min and then resuspended in 5 ml of 0.05 KPO_4 buffer, pH 8.0. The partially purified virus preparations were layered onto preformed 20–35% (w/w) CsCl suspensions prepared in 0.05 M KPO_4 buffer, pH 8.0. After centrifugation at 120,000 *g* for 5 hr, the virus bands located approximately 12 mm from the bottom of the 5.5-ml tubes, were collected with a syringe and diluted in 0.05 M KPO_4 buffer, pH 8.0, to a final volume of 10 ml. The viruses were concentrated by centrifugation at 84,500 *g* for 2 hr and finally resuspended in 1 ml of 0.05 M KPO_4 buffer, pH 8.0.

The ultraviolet absorption spectra of purified virus preparations were obtained with a Beckman DU 50 spectrophotometer in the 240–360 nm range. An extinction coefficient of 2.4 (10) was used to calculate virus concentration.

Electrophoresis. Purified virus preparations were analyzed in 10% polyacrylamide gels containing SDS as described by Weber and Osborn (19). Samples were dissociated by adding two volumes of a solution containing 0.1 ml of electrophoresis $NaPO_4$ buffer, 0.25 ml of 10% SDS, 25 μl of 2-mercaptoethanol, and 0.25 ml of 60% sucrose, and boiling the mixture for 1 min. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and cowpea mosaic virus coat proteins (22 and 42 kDa) were used as markers for molecular weight determinations.

Serology. Antisera to the *A. pintoi* and *Stylosanthes* sp. viruses were prepared by injecting New Zealand white rabbits with purified virus preparations standardized to a concentration of 1 mg/ml. A series of four injections was given at weekly intervals, following the foot pad technique of immunization (20). Each injection consisted of 0.15 ml of the virus emulsified with an equal volume of complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. The antisera were collected at weekly intervals for 1 mo after the last injection.

Antisera to bean common mosaic virus, peanut mottle virus, peanut stripe virus, soybean mosaic virus, and watermelon mosaic virus 2 were obtained either at CIAT or the Plant Pathology Department, University of Florida.

The Ouchterlony, DAS-ELISA, and serologically specific electron microscopy (SSEM) tests were conducted as described by Purcifull and Batchelor (15), Clark and Adams (3), and Derrick (5), respectively. A monoclonal antibody specific to the potyvirus group (Agdia, Inc. Elkhart, IN) was also used in this investigation.

RESULTS

The causal agents of the *A. pintoi* ring spot and *Stylosanthes* sp. chlorosis and leaf malformation diseases were manually transmitted to seedlings of the bean cultivar Bountiful, inducing chlorotic local lesions on the inoculated primary leaves. However, only the *Stylosanthes* sp. virus induced noticeable systemic symptoms (severe mosaic and leaf malformation) in Bountiful bean.

An electron microscopic examination of symptomatic *A. pintoi*, *Stylosanthes* sp., and inoculated bean plants demonstrated the presence of filamentous flexuous virus particles, measuring about 750 nm in length and 15 nm in diameter (Fig. 2A and B).

The virus isolated from *A. pintoi* was also manually transmitted to the following legumes: *A. hypogaea* (21 different genotypes), *Canavalia* sp., *S. occidentalis*, *C. brasilianum*, seven soybean cultivars (Clark, ICA L-121, Mandarin, Marshall, Ogden, Rampage, and York), three bean cultivars (Dubbele Witte, Widusa, and Black Turtle Soup), *P. lunatus*, pea, *S. capitata*, *S. macrocephala*, *V. radiata*, and *V. u. unguiculata* 'Blackeye' (Table 1). Other species systemically infected by the *A. pintoi* virus were *N. benthamiana* and *P. angulata*. The list of insusceptible and local lesion hosts is shown in Table 1. The virus isolated from *Stylosanthes* sp. systemically infected peanut, five bean cultivars (Dubbele Witte, Stringless Green Refugee, Redlands Greenleaf C, Black Turtle Soup, and Great Northern 123), pea, *S. macrocephala*, and *V. unguiculata* (cv. Blackeye). The only nonleguminous species infected systemically was *N. benthamiana* (Table 1). The list of local lesion

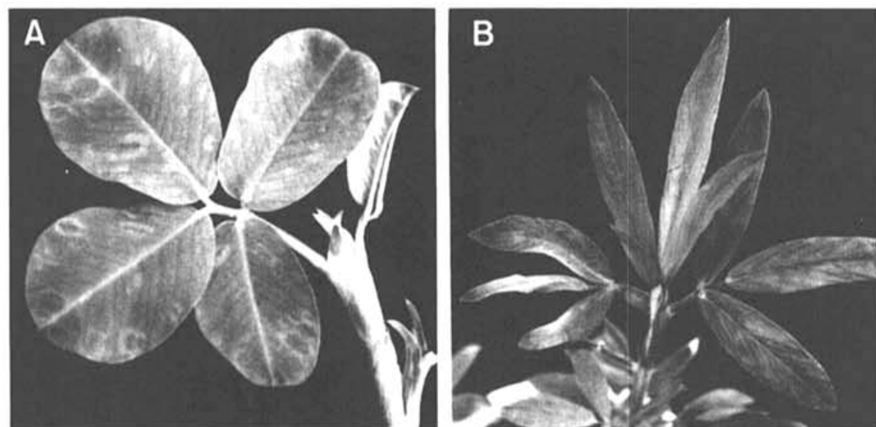

Fig. 1. (A) Foliar ring spot symptoms induced by a potyvirus related to peanut mottle virus in the tropical forage legume *Arachis pintoi* and **(B)** foliar chlorosis and malformation symptoms induced by a potyvirus related to peanut mottle virus in the tropical forage legume *Stylosanthes macrocephala*.

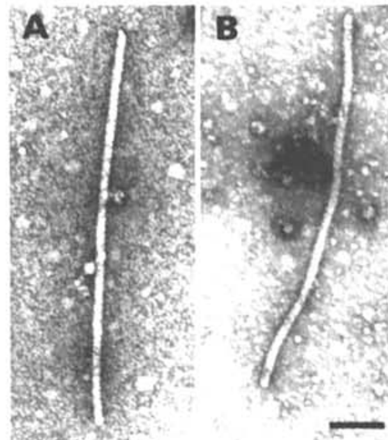

Fig. 2. Filamentous flexuous virus particles observed in leaf extracts of **(A)** *Arachis pintoi* and **(B)** *Stylosanthes* sp. plants affected by foliar ring spot and chlorosis, respectively. Scale bar = 100 nm.

and insusceptible hosts can be found in Table 1. All of the above results were confirmed by serology.

Both the *A. pintoi* and *Stylosanthes* sp. viruses were transmitted by *M. persicae* to the cowpea and bean test plants, respectively, as demonstrated by symptom expression and serological tests.

The ELISA tests of the *A. pintoi* and *Stylosanthes* spp. seeds, using their respective virus antisera, did not detect the presence of the homologous viruses in any of the seeds tested. In neither case, however, was the incidence of these viruses determined in the fields where the test seed was collected.

Thin tissue sections of *A. pintoi* and *S. macrocephala* plants infected with the respective *Arachis* and *Stylosanthes* virus isolates were shown by electron microscopy to contain cylindrical inclusions consisting of pinwheels and short, curved laminated aggregates (Fig. 3A and B). Despite the similarity between the short, curved laminated aggregates observed in these sections and those induced by subdivision IV potyviruses (7), no scrolls (tubes in longitudinal section), which are also characteristic of subdivision IV cytoplasmic inclusions, were found.

The *Arachis* and *Stylosanthes* viruses were purified from their respective propagation hosts in quantities of 10 and 7.5 mg, respectively, per kilogram of infected tissue. Absorbance 260/280 values for these purified preparations ranged between 1.25 and 1.4, uncorrected for light scattering.

Electrophoretic analyses of these purified preparations in the presence of SDS yielded single-coat protein subunits of approximate molecular weight 34 kDa for both the *Arachis* and *Stylosanthes* viruses.

A precipitin reaction of identity was observed in Ouchterlony tests between peanut mottle virus (PMoV) and the *A. pintoi* virus in reciprocal tests. The PMoV antiserum also detected the *Stylosanthes* sp. virus in infected tissue extracts and purified preparations without the formation of spurs over adjacent PMoV precipitin reactions. Neither the *A. pintoi* virus nor the *Stylosanthes* virus was antigenically related to bean common mosaic virus, peanut stripe virus, soybean mosaic virus, or watermelon mosaic virus 2 in any of the serological tests performed in this study.

In SSEM tests, the antiserum to PMoV trapped approximately three and four times the average number of particles of the *Stylosanthes* and *A. pintoi* viruses, respectively, observed in untreated leaf dips (265 particles per 1,000 μm^2). No virus particle trapping occurred in these tests when soybean mosaic virus was included as control. Antisera to the *A. pintoi* and *Stylosanthes* viruses trapped 2,072 and 1,169 particles per 1,000 μm^2, respectively, in homologous SSEM tests, and 1,169 and 790 particles per 1,000 μm^2, respectively, in heterologous tests.

The commercial anti-potyvirus monoclonal antibody detected bean common mosaic and soybean mosaic potyviruses but did not react with either the *A. pintoi* or *Stylosanthes* viruses in ELISA tests after standard overnight incubation periods (16 hr) of the IgG conjugate. However, when this incubation time was extended to 26 hr, a positive reaction was obtained with the potyvirus isolated from *Stylosanthes* sp. (A_{405nm} of 1.8 30 min after addition of substrate) and, to a marginal extent (A_{405nm} of 0.25 30 min after addition of substrate), with the *A. pintoi* potyvirus. (A_{405nm} values for extracts of healthy plants ranged from 0 to 0.1 at the above-mentioned final reading time.)

Table 1. Comparative host range of two potyviruses isolated from the forage legumes *Arachis pintoi* and *Stylosanthes* sp., in Colombia, South America

	Local/systemic symptoms[a]	
Plant species	**APP[b]**	**STP[c]**
Leguminosae		
Arachis hypogaea	–/MT	–/MT
A. pintoi	–/RS	–/–
Canavalia sp.	–/MS	NT
Senna occidentalis	–/MS	–/–
Centrosema brasilianum	–/MS	NT
Glycine max	LL/MT	–/–
Phaseolus vulgaris	LL/MS,N	LL/MS,N
P. lunatus	–/MT	NT
Pisum sativum	–/MS	–/MS
Stylosanthes macrocephala	–/MM	–/MM
S. capitata	–/MM	–/–
Vigna radiata	–/SS	–/–
V. unguiculata subsp. *unguiculata*	–/MS	–/MS
Other plant families		
Chenopodium quinoa	–/–	–/–
C. amaranticolor	LL/–	LL/–
Datura stramonium	–/–	–/–
Gomphrena globosa	–/–	–/–
Nicotiana benthamiana	–/MM	–/MM
N. glutinosa	–/–	–/–
N. tabacum	–/–	–/–
Physalis angulata	–/MM	–/–

[a] LL = local lesions, MM = mild mosaic, MS = mosaic, MT = mottle, N = necrosis, NT = not tested, SS = symptomless, – = no infections determined by ELISA or electron microscopy test.
[b] APP = *Arachis pintoi* potyvirus.
[c] STP = *Stylosanthes* sp. potyvirus.

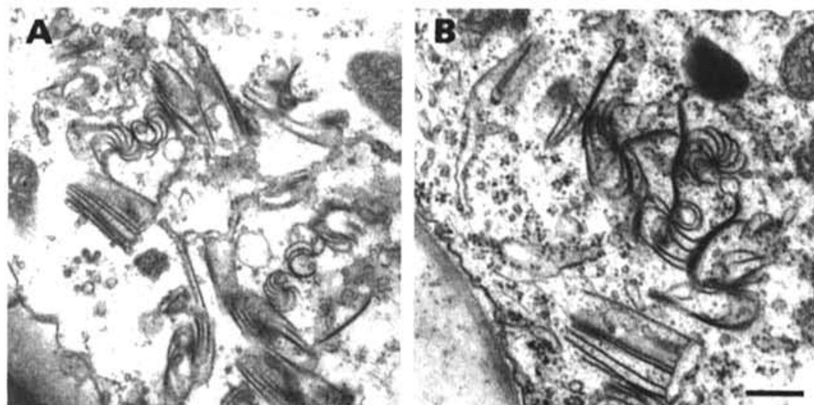

Fig. 3. Cytoplasmic cylindrical inclusions induced by **(A)** a potyvirus isolated from *Arachis pintoi* and **(B)** another potyvirus isolated from *Stylosanthes* spp., consisting of pinwheels and short, curved laminated aggregates. Scale bar = 500 nm.

DISCUSSION

Considering their particle morphology, the formation of cytoplasmic cylindrical inclusions in infected plant cells, and the molecular weight of their respective capsid protein subunit, we conclude that the two viruses isolated in this investigation from *A. pintoi* and *Stylosanthes* sp. are members of the potyvirus group (10). The serological relationship demonstrated here between these two viruses and a known member of the potyvirus group, peanut mottle virus (2), further supports this conclusion.

The failure of the anti-potyvirus monoclonal antibody to detect these aphid-transmitted legume potyviruses has also been observed in ELISA tests conducted by two independent laboratories in the United States, using the same potyviruses tested here (G. I. Mink, *personal communication*), and a strain of PMoV (12). However, this study has shown that longer incubation periods of the IgG conjugate may circumvent the virus detection problems reported in tests of the "universal" monoclonal antibody with some aphid-transmitted potyviruses, which probably possess altered amino acid sequences of the epitope recognized by the commercial monoclonal antibody.

The close serological relationship observed between the *A. pintoi* and *Stylosanthes* sp. potyviruses and PMoV, their pathogenic reactions in several peanut genotypes, and the formation of cytoplasmic inclusions similar to those induced by PMoV (6) suggest that the two tropical forage legume potyviruses isolated in this study are closely related to PMoV despite some differences, particularly with regard to the host range of the *Stylosanthes* sp. potyvirus. The observation of differences in host range among PMoV strains, however, is not unique to this study (1,9,14,18) and is probably related to the lack of a universal set of differential peanut cultivars or diagnostic hosts tested with representative PMoV strains from different parts of the world. Furthermore, PMoV is a known pathogen of other forage legumes (4).

Possible transmission of these legume potyviruses in the seed of *A. pintoi* or *Stylosanthes* spp. can not be excluded, because the actual incidence of these viruses in the localities where most of the seed tested in this investigation was produced was not determined. Also, the relatively low seed-transmissibility of PMoV in peanut (2) may explain the negative results obtained in the ELISA test of the 280 *A. pintoi* seeds harvested from plants affected with ring spot. Moreover, *A. pintoi* is also vegetatively propagated (8) and, consequently, it is important to test for the absence of these potyviruses in vegetative and/or sexual seed of *A. pintoi* and *Stylosanthes* spp. before these promising tropical forage legumes become widely distributed in the tropics.

ACKNOWLEDGMENTS

We thank Ana Maria Piedrahita and Andrea Niessen for their technical assistance in the initial virus characterization work and Guillermo Guzman for his assistance in the preparation of the photographic material. We also thank Gloria I. Lopez for the preparation of this manuscript.

LITERATURE CITED

1. Bock, K. R. 1973. Peanut mottle virus in East Africa. Ann. Appl. Biol. 74:171-179.
2. Bock, K. R., and Kuhn, C. W. 1975. Peanut mottle virus. No. 141 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England. 4 pp.
3. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
4. Demski, J. W., Kahn, M. A., Wells, H. D., and Miller, J. D. 1981. Peanut mottle virus in forage legumes. Plant Dis. 65:359-362.
5. Derrick, K. S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56:652-653.
6. Edwardson, J. R., and Christie, R. G. 1986. Potyviruses. Pages 368-464 in: Viruses Infecting Forage Legumes. Vol. 2. Fla. Agric. Exp. Stn. Monogr. 14.
7. Edwardson, J. R., Christie, R. G., and Ko, N. J. 1984. Potyvirus cylindrical inclusions—Subdivision-IV. Phytopathology 74:1111-1114.
8. Grof, B. 1985. Forage attributes of the perennial groundnut *Arachis pintoi* in a tropical savanna environment in Colombia. Pages 168-170 in: Proc. Int. Grassl. Congr., 15th.
9. Herold, F., and Munz, K. 1969. Peanut mottle virus. Phytopathology 59:663-666.
10. Hollings, M. 1981. Potyvirus group. No. 245 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England. 7 pp.
11. Lenné, J. M., and Calderon, M. A. 1985. Disease and pest problems of *Stylosanthes*. Pages 279-293 in: The Biology and Agronomy of *Stylosanthes*. H. M. Stace and L. A. Edye, eds. Academic Press, New York.
12. Li, R. H., Zettler, F. W., Elliot, M. S., Petersen, M. A., Still, P. E., Baker, C. A., and Mink, G. I. 1991. A strain of peanut mottle virus seed-borne in bambarra groundnut. Plant Dis. 75:130-133.
13. Morales, F. J., Niessen A., Ramirez, B., and Castaño, M. 1990. Isolation and partial characterization of a geminivirus causing bean dwarf mosaic. Phytopathology 80:96-101.
14. Paguio, O. R., and Kuhn, C. W. 1973. Strains of peanut mottle virus. Phytopathology 63:976-980.
15. Purcifull, D. E., and Batchelor, D. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions. Tech. Bull. 788. Univ. Fla. Inst. Food Agric. Sci. 39 pp.
16. Schultze-Kraft, R., Reid, R., Williams, R. J., and Coradin, L. 1985. The existing *Stylosanthes* collections. Pages 125-146 in: The Biology and Agronomy of *Stylosanthes*. H. M. Stace and L. A. Edye, eds. Academic Press, New York.
17. Stace, H. M., and Edye, L. A., eds. 1984. The Biology and Agronomy of *Stylosanthes*. Academic Press, New York. 636 pp.
18. Sun, M. K. C., and Hebert, T. T. 1972. Purification and properties of a severe strain of peanut mottle virus. Phytopathology 62:832-839.
19. Weber, K., and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.
20. Ziemiecki, A., and Wood, K. R. 1975. Serological demonstration of virus-specific proteins associated with cucumber cotyledons. Physiol. Plant Pathol. 7:171-177.