Cowpea Stunt: A Disease Caused by a Synergistic Interaction of Two Viruses

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ABSTRACT

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Severely stunted cowpea plants have been found in Georgia fields in each of the last 4 yr, 1974 to 1977. Leaves of the stunted plants were small, mottled, blistered, and malformed. The causal agent was sap-transmitted to cowpeas. Several other hosts were susceptible and back inoculation from *Cucumis sativus* and *Cassia obtusifolia* to cowpeas established the presence of two viruses: cucumber mosaic virus (CMV) from *C. sativus* and a potyvirus, later identified as blackeye cowpea mosaic virus (BlCMV), from *C. obtusifolia*. When seedlings of California Blackeye cowpeas were inoculated simultaneously with CMV and BlCMV, a strong synergistic reaction occurred; the primary and first two trifoliolate leaves became necrotic within 5-10 days and usually abscised. Subsequent leaves were severely diseased but free of the necrosis; the symptoms then appeared similar to those observed on field plants. When inoculated singly, each virus caused a relatively mild disease; leaves had a mild mottle, and plants displayed moderate stunting during the early infection period and almost no stunting at senescence. In a greenhouse study, CMV reduced yield of California Blackeye seed by 14.2% and BlCMV by 2.5%. Yield on doubly infected plants, however, was reduced 86.4%. Furthermore, the double infection reduced leaf weight, stem weight, and root weight by 94.3, 89.3, and 87.3%, respectively. Seed and aphid transmission studies showed that the viruses can be transmitted from doubly-infected plants to cause single or double infections in cowpeas. The name cowpea stunt is proposed for the disease caused by the synergistic interaction of CMV and BlCMV.

Additional key words: serology, nonpersistent transmission.

Virus diseases of plants caused by simultaneous infection of two or more unrelated viruses have been recognized since the 1920's (6, 17, 19, 20). More recent examples include corn lethal necrosis caused by maize chlorotic mottle virus in combination with either maize dwarf mosaic virus or wheat streak mosaic virus (18), and a disease complex of cowpeas, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*, caused by Moroccan strains of cucumber mosaic virus (CMV) and cowpea aphid-borne virus (CAMV) (8, 9).

In a preliminary report (22), we noted a severe stunting disease of cowpeas caused by CMV and an unknown virus now identified as blackeye cowpea mosaic virus (BlCMV). This paper is concerned with the characterization and naming of the disease, identification of the specific viruses involved, synergistic effects of the two viruses on plant growth and seed yield, and transmission of the viruses via seed and aphids.

MATERIALS AND METHODS

Virus maintenance.—Sap inoculations were made by grinding diseased leaf tissue (1 g /9 ml) in neutral potassium phosphate buffer (0.01 M) containing celite (1%) and rubbing primary leaves of 8- to 9-day-old cowpea plants with a cheesecloth pad soaked in the prepared inoculum. Viruses in either single or mixed infections were maintained in California Blackeye cowpeas (previously named Early Ramshorn by the seed source, W. Atlee Burpee Co., 300 Park Avenue, Warminster, PA 18974). Test plants were kept in a greenhouse (24 to 30 C) in 10- or 15-cm diameter pots containing a mixture of soil, sand, and vermiculite (2:1:1, v/v) provided with a complete fertilizer.

Identification studies.—Both CMV and BlCMV were partially purified for identification studies. For CMV, diseased tissue (1 g/ml of buffer) was homogenized in 0.5 M phosphate buffer (pH 8) containing 0.5% mercaptoethanol, and the resulting sap was clarified with butanol (8%); the virus was concentrated with one cycle of ultracentrifugation, and test virus was fractionated after centrifugation on sucrose gradients (10-40%) in 0.01 M phosphate buffer (pH 8). Purification of BlCMV was achieved by the method described by Lima et al. (13).

Sedimentation coefficient values were estimated (4) in sucrose gradients by using cowpea chlorotic mottle virus (CCMV) ($S_{20,w}$ = 88) and southern bean mosaic virus (SBMV) ($S_{20,w}$ = 115) as markers. Virus preparations were negatively stained with phosphotungstic acid (2%) for observation with the electron microscope. For serological tests, CMV and BlCMV antisera were furnished by the Plant Virus Laboratory, University of Florida,

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Gainesville, FL 32611. Immunodiffusion tests were conducted in petri dishes containing agarose (0.8%) and NaN_3 (1%); tests with BlCMV also included sodium dodecyl sulfate (0.5%).

Plant growth and yield studies.—For plant growth studies, California Blackeye plants (one per pot) were grown in 15-cm diameter pots. The treatments were CMV, BlCMV, CMV plus BlCMV, and buffer-rubbed control, each with 12 replications arranged in a randomized block design on a greenhouse bench where temperature ranged from 24 to 30 C. Plants were inoculated 9 days after seeding, and plant measurements were made 3 wk after inoculation. Dry weights of leaves, stems, and roots were determined by drying the tissue to a constant weight at 80 C. Plants were grown similarly to determine yield of seed except they were maintained until senescence. Seed were dried at 22 to 25 C until the weight remained constant for 10 days.

Transmission studies.—Seed transmission of CMV and BlCMV was determined with seed from cowpea cultivars California Blackeye and Knuckle Purple Hull inoculated 9 days after seeding. For aphid transmission studies, *Myzus persicae* (Sulz.) was cultured on Chinese cabbage (*Brassica chinensis* L.). After 12 hr in a glass petri dish, apterous aphids were allowed an acquisition access for up to 60 sec on California Blackeye plants infected with CMV, BlCMV, or CMV plus BlCMV. Then, groups of five aphids were transferred to individual plants of California Blackeye and Knuckle Purple Hull; 12 hr later the aphids were killed with aldicarb.

Plants in the transmission studies were observed for 3 wk, and those with symptoms were checked for virus identity based on symptoms produced on California Blackeye as follows: (i) CMV alone caused local necrosis in 2-3 days and mild mottle systemic symptoms, (ii) BlCMV alone caused local chlorosis and necrosis in 5-7 days and mild mottle systemic symptoms, and (iii) CMV plus BlCMV together caused stem and vein necrosis and severe stunting.

RESULTS

Symptomatology.—Severely stunted cowpea plants were found in commercial fields in widely separated areas in Georgia during 1974-1977. The plants had small, mottled, blistered, and malformed leaves (Fig. 1-A). Discoloration varied from different shades of green to green and yellow combinations, particularly with yellowing associated with major veins. The number of leaves was reduced about 30%, and internodes were shortened 50% or more. Pod set was poor, and the number of seed per pod was reduced 50-75%.

Sap inoculation studies demonstrated that the disease symptoms observed in the field could be reproduced on several cowpea cultivars in the greenhouse. However, a striking initial shock reaction (acute phase) was observed on California Blackeye plants. About 5 to 7 days after inoculation of 8- to 9-day-old seedlings, epinasty of the trifoliolate leaves was followed by necrosis which developed on the main stem immediately below the primary leaves, on petioles and petiolules of trifoliolate leaves, and on veins of trifoliolate leaves. The inoculated primary leaves and the first trifoliolate leaves usually abscised, and infrequently, the plant died (Fig. 1-B). On

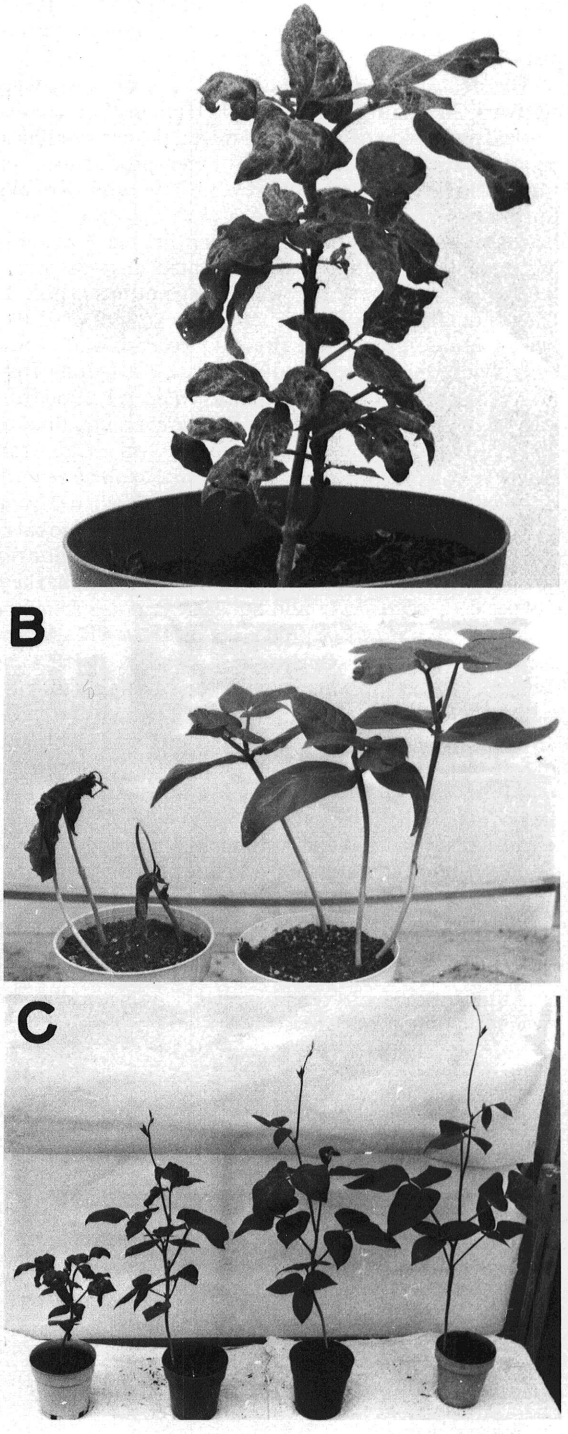

Fig. 1-(A to C). California Blackeye cowpea plants infected singly and doubly with cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV). **A)** Severe mosaic and stunting of plant doubly infected for 7 wk. **B)** A strong synergistic reaction caused death of plants on the left (12-15 days after inoculation); plants on right are infected with BlCMV only. **C)** Comparison of plant size at 3 wk after inoculation; from left to right CMV + BlCMV, CMV only, BlCMV only, and buffer-rubbed control.

surviving plants, new leaves developed which were free of necrosis, but the plants were severely diseased (chronic phase), similar to the plant in Fig. 1-A.

The acute disease phase did not develop on cowpea cultivars Clay and Knuckle Purple Hull; neither necrosis nor leaf abscission occurred. However, the chronic phase, from 3 wk after inoculation until seed production, was similar for California Blackeye, Clay, and Knuckle Purple Hull.

Isolation of two viruses.—When a limited host range was inoculated with sap from stunted cowpea plants, species of several genera developed symptoms (Table 1). Back inoculation to California Blackeye indicated that two or more viruses were present. Necrosis and typical field symptoms were produced with inoculum from several cultivars of bean (*Phaseolus vulgaris* L.) and from lima bean (*Phaseolus lunatus* L.). However, mild mottle, nonstunting, symptoms developed on California Blackeye cowpeas with inoculum from *Cassia obtusifolia* L., cucumber (*Cucumis sativus* L.), soybeans (*Glycine max* L. Merr.), squash (*Cucurbita pepo* L.), and tobacco (*Nicotiana tabacum* L.). Sap from six combinations of hosts caused necrosis and stunting on California Blackeye cowpeas: *C. obtusifolia* and squash, *C. obtusifolia* and tobacco, *C. obtusifolia* and cucumber, soybeans and cucumber, soybeans and squash, and soybeans and tobacco. Based on our experience, a highly reliable method to demonstrate the presence of two viruses in the stunted cowpea plants is illustrated in Fig. 2. Although any of several cucumber, squash, and tobacco cultivars could be used for isolation of CMV, we prefer *C. obtusifolia* rather than soybeans for BlCMV isolation because obvious local and systemic symptoms develop on *C. obtusifolia* and only mild systemic ones on soybeans, a host which is sometimes difficult to infect.

Identification of viruses.—Symptoms on cowpeas caused by the virus from cucumber, squash, and tobacco resembled those caused by CMV in previous studies (11) in Georgia. This identification was confirmed by serological tests, an estimated sedimentation coefficient ($S_{20,w}$) of 96 to 98, host range, and physical properties in

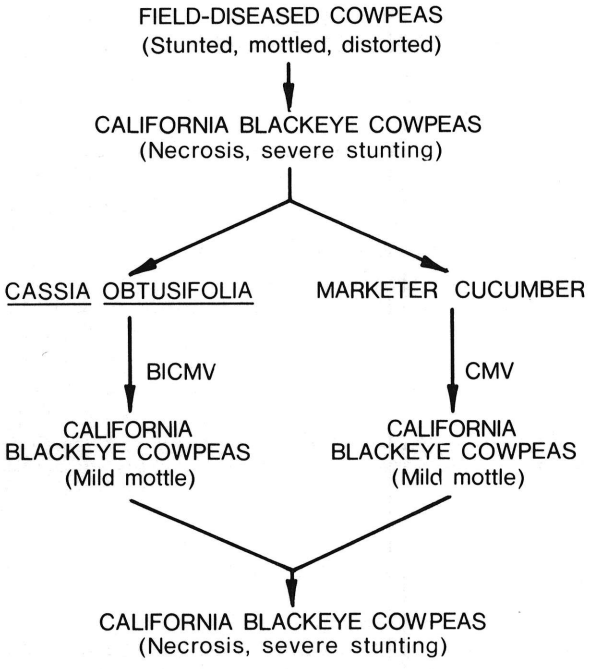

Fig. 2. A method for the separation of the two viruses, cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV), which cause cowpea stunt.

TABLE 1. Reaction of several test hosts to single and double inoculations (mechanical) with cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV)

Test hosts	Local and systemic symptoms[a]		
	CMV	BlCMV	CMV + BlCMV
Arachis hypogaea	I	I	I
Cassia obtusifolia	I	LNL,SM	LNL,SM
Cucumis sativus			
'Dixie', 'Marketer'	SM	I	SM
Cucurbita pepo			
'Crookneck', 'Straightneck'	SM	I	SM
Glycine max			
'Bragg', 'Davis'	I	SM	SM
Nicotiana tabacum			
'Havana 423', 'Hicks'	SM	I	SM
Phaseolus lunatus			
'Henderson'	SM	SM	SM
Phaseolus vulgaris			
'Bountiful', 'Pinto', 'Roma'	SM	LC[b]	LC,SM
Vigna unguiculata			
'California Blackeye'	LCN,SM	SM[c]	LCN,SN,SM, St[d]
'Knuckle Purple Hull', 'Clay'	LC,SM	LC,SM	LC,SM,St

[a]Letter designations: I = immune, LC = local chlorosis, LN = local necrosis, LNL = local necrotic lesion, SM = systemic mottle, SN = systemic necrosis, and St = stunt.

[b]Symptomless systemic reaction.

[c]Occasional chlorotic and necrotic spots on inoculated leaves; also occasional limited necrosis of stem and petioles.

[d]Sometimes the plants died.

sap (thermal inactivation point between 50 and 55 C, dilution end point between 10^{-3} and 10^{-4}, and longevity in vitro between 1 and 2 days at 22 C).

The virus from *C. obtusifolia* (Fig. 2) was cultured in California Blackeye cowpeas. Electron microscope observations of partially purified preparations revealed flexuous rod particles with an average length of 760 nm. In sucrose gradients, the estimated sedimentation coefficient ($S_{20,w}$) was 150-155. Physical properties in sap were as follows: thermal inactivation point between 55 and 60 C, dilution end point between 10^{-3} and 10^{-4}, and longevity in vitro between 1 and 2 days at 22 C. These characteristics suggested that the virus was the same or similar to the BlCMV being worked with in Florida (2, 7, 23, 24). Indeed, immunodiffusion serology tests demonstrated identity, or at least similarity, between BlCMV from Florida and the *Cassia* isolate from Georgia. Other serological tests (J. A. A. Lima, and D. E. Purcifull, *unpublished*) have demonstrated that BlCMV is serologically related to, but distinct from, an East African strain of CAMV in SDS-double immunodiffusion tests.

Synergistic reaction.—The disease reaction caused by the mixed infection with CMV and BlCMV on California Blackeye cowpeas was clearly distinct from the relatively mild diseases caused by each virus alone. Plant size reduction is illustrated in Fig. 1-C. When measurements were made 3 wk after inoculation, single infections of CMV and BlCMV caused some reduction of most plant parts (Table 2). The mixed infection, however, was synergistic in that it caused reductions greater than the additive effects of the single infections for plant height, leaf number, leaf weight, and stem weight. Synergistic effects on root weight could not be determined because the additive reductions caused by single infections were greater than the total root weight of control plants.

The reduced plant growth caused by single infections had relatively little effect on yield; pod number, seed number, and seed weight were not reduced significantly by either CMV or BlCMV (Table 2). However, drastic losses were noted for the mixed infection. Yield loss was due primarily to fewer pods (73-77%), but also fewer seed were produced per pod (13-37%) and smaller seed (16-24%) also were produced on doubly-infected plants.

Transmission characteristics.—Both CMV and BlCMV were transmitted through the seed of two cowpea cultivars, California Blackeye and Knuckel Purple Hull (Table 3). A few seed from doubly-infected plants had both viruses. The frequency of seed transmission was similar regardless of single or double infections.

Myzus persicae transmitted both CMV and BlCMV from both single and mixed infections in a nonpersistent manner. From a mixed infection, the aphids transmitted the viruses singly and in combination (Table 3).

DISCUSSION

Mixed infections of unrelated viruses are relatively common in plants in nature. In Martyn's annotated lists (15, 16) of plant virus names, more than 50 diseases are

TABLE 2. The effect of single and double infections of cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV) on plant growth and yield of California Blackeye cowpeas in the greenhouse[x]

Treatment	Growth[y]					Yield		
	Plant height (cm)	Leaf		Stem wt (g)	Root wt (g)	Pod number	Seed number	Seed wt (g)
		Number	Wt (g)					
Control	67.0 a[z]	6.7 a	3.67 a	2.53 a	1.90 a	13.3 a	78.8 a	16.2 a
CMV	44.9 b	6.3 a	2.54 b	1.23 b	1.10 b	14.9 a	64.0 a	13.9 a
BlCMV	58.8 a	5.9 a	2.54 b	1.58 b	0.75 b	12.5 a	69.7 a	15.8 a
CMV + BlCMV	22.3 c	4.3 b	0.21 c	0.27 c	0.24 c	3.4 b	12.7 b	2.2 b

[x]Plants were inoculated 9 days after seeding; 12 plants/treatment; all values are the average per plant.
[y]Measurements were made 3 wk after inoculation.
[z]Treatment means followed by different letters in the same column are significantly different at $P = 0.05$, according to the Scheffé test.

TABLE 3. Transmission of cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV) via the seed of two cowpea cultivars and by the aphid, Myzus persicae

Transmission via	Percentage of virus transmission[a, b]				
	From single infection		From double infection		
	CMV	BlCMV	CMV	BlCMV	Both
Seed:					
California Blackeye	10.9	6.3	7.0	3.2	3.6
Knuckle Purple Hull	3.4	5.4	1.3	7.4	0.7
Aphid	22.8	17.1	13.8	15.8	26.7

[a]Frequency of seed transmission was determined by observation of 546, 545, and 539 California Blackeye plants and 297, 294, and 296 Knuckle Purple Hull plants for CMV, BlCMV, and virus combination, respectively.
[b]Aphid transmission was determined by observation of 76, 89, and 98 cowpea plants for CMV, BlCMV, and the virus combination, respectively. Five aphids were placed on each test plant.

cited that may be caused by concurrent infection by two or more different viruses. Frequently, distinct disease names are used to distinguish the mixed infection disease from diseases caused by the individual viruses. Therefore, we propose the name cowpea stunt for the cowpea disease caused by the combination CMV and BlCMV for three reasons. First, cowpea stunt does not resemble, and would not be confused with, the relatively mild diseases caused by CMV and BlCMV. Second, cowpea stunt appears to be an economically important disease that has been found repeatedly in Georgia, and both CMV and BlCMV were found in the same cowpea field in Florida (1, 2). Third, cowpea stunt symptoms are substantially different from those caused by four other mixed infections of viruses in cowpeas which have been found in nature: CMV and bean yellow mosaic virus (BYMV) (10), CMV and CCMV (10), CCMV and the cowpea strain of SBMV (12), and CCMV and BYMV (10).

There are many reports which indicate that mixed virus infections cause synergistic reactions. Frequently, viral combinations cause various degrees of necrosis, as noted by Vanterpool (20) for tobacco mosaic virus and potato virus X (PVX) which cause the tomato streak disease. In another classic example, however, Smith (19) found that PVX and potato virus Y cause rugose mosaic of potatoes, a disease with little or no necrosis. In cowpeas doubly-infected with CMV and BlCMV, a necrotic or non-necrotic reaction is dependent on the cultivar. The necrotic reaction in cowpeas simply may be an initial shock reaction since plants recover from the necrosis 2 to 3 wk after inoculation.

The identity of the two virus components of cowpea stunt does not seem to be in question; CMV has been found in cowpeas on numerous occasions (1, 9, 10, 11), and its intrinsic properties are well-established. Also, the biological and serological properties of BlCMV are relatively clear; only the name is in question. In 1955, Anderson (2) described a cowpea disease and named the pathogen BlCMV; the new name was based on symptomatology, host range, physical properties in sap, and transmission characteristics. In 1966, Lovisolo and Conti (14) determined some biophysical properties of a cowpea virus and named it CAMV. Later, Martyn (15) and Bock and Conti (3) considered BlCMV and CAMV to be similar or synonymous. Based on studies conducted in Florida (7, 13, 23, 24) and on our observations, we believe BlCMV and CAMV should be considered closely related, but each should maintain an identity with a distinct name, at least until direct comparisons can be made.

Fischer and Lockhart (9) reported that Morrocan strains of CMV and CAMV cause a synergistic effect on Early Ramshorn cowpeas. Although the synergism classification was based on observations only, the reaction was probably similar to the one we found with CMV and BlCMV on California Blackeye cowpeas. This provides additional evidence for a relationship between BlCMV and CAMV.

Name confusion also exists between BlCMV and BYMV (24). Two studies (5, 13) demonstrated some degree of serological relationship between the two viruses, and a cowpea disease reported to be caused by BYMV in Alabama (10) and Georgia (11) had symptoms similar or identical to those caused by BlCMV. However, Edwardson et al. (7) demonstrated marked differences between the inclusion bodies induced by BlCMV and three BYMV isolates from hosts other than cowpeas. With the assistance of J. A. A. Lima and R. G. Christie of the University of Florida, we established that the Georgia isolate (11) (virus obtained from seed produced in 1959) was serologically similar or identical to BlCMV, induced inclusion bodies like BlCMV and none like BYMV, and caused a typical synergistic reaction with necrosis on California Blackeye cowpeas in a mixed infection with CMV. Therefore, we conclude that the Georgia isolate, formerly designated BYMV, should be named BlCMV. Unfortunately, a culture of the Alabama isolate (10) is not available, and a decision about its identity cannot be made. However, results of the Alabama study (10) demonstrated that the possibility of a virus distinct from BlCMV persists; when Early Ramshorn (now named California Blackeye) and Clay cowpeas were inoculated simultaneously with CMV and BYMV, neither necrosis nor synergism were observed.

The potential economic importance of cowpea stunt is greatly increased because of the transmission characteristics of the two virus components. Both are transmitted through seed and by the same aphid species in both single and double combinations. Although critical studies were not conducted, the frequency of aphid transmission of each virus was about twice as great from a mixed infection as from a single infection. Thus, the total percent transmission from doubly infected plants was 40.5% for CMV and 41.5% for BlCMV while the respective percentages from singly infected plants were 22.8 and 17.1. This increased transmission could be epidemiologically significant. Furthermore, stunting (synergism) can occur when either of the viruses is established in cowpeas for at least 1 wk before infection with the second virus (Pio-Ribeiro, *unpublished*). These dual transmission characteristics are not common to most plant diseases caused by mixed virus infections. For example, the cowpea disease caused by CCMV and SBMV (12) occurs only occasionally in nature; both viruses may be transmitted by the same beetle (21) but beetle transmission efficiency is poor and only SBMV is seed-transmitted.

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