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Purification and Serology of an Isolate of Zucchini Yellow Mosaic Virus[1]

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Abstract: An isolate of zucchini yellow mosaic virus, ZYMV-7, was purified and its antiserum produced. Systemic leaves from inoculated zucchini plants taken 9-10 days after inoculation had the highest virus concentration, and were used in virus purification. Yields of purified virus estimated by spectrophotometer were 18-22 mg/100g leaf tissues. The O. D. ratio of A260/280 from several separate preparations ranged from 1.27 to 1.31. The purified virus preparation obtained from the first isopycnic centrifugation in cesium sulfate was found contaminated with host proteins, however, the contaminated materials could be removed by a second isopycnic centrifugation. The reciprocal titers of antiserum against homologous antigens in crude leaf extracts and purified virus preparations were 2048 and 4096, respectively, in ring interface precipitin tests. The titer of this antiserum against crude sap antigens was 16 in SDS-immunodiffusion tests. Antisera collected at several bleedings reacted with host constituents weakly, but the addition of 2% bovine serum albumin to the antisera or to agar medium completely removed the non-specific reactions in SDS-gel diffusion tests. Antiserum to ZYMV-7 produced precipitin bands with all 10 ZYMV isolates, i. e., ZYMV-1 to ZYMV-9 from Taiwan, and ZYMV-FL from Florida. There were no serological relationships of ZYMV-7 with WMV-1 and WMV-2 (Florida isolate). However, an antiserum to WMV-2, obtained from University of Florida, reacted with ZYMV isolates strongly but formed definite spurs with WMV-2 in SDS-gel diffusion tests, indicating that ZYMV isolates from Taiwan are different from WMV-2 Florida isolate but have some serological relations to certain types of WMV-2.
(Key words: purification, serology, zucchini yellow mosaic virus)

Introduction

More than 25 viruses, including at least 7 belonging to potyvirus group, are found naturally in cucurbits (16,22). Among them zucchini yellow mosaic virus (ZYMV) first described by Lisa et al. in 1981 from Italy is a relatively new potyvirus (15). The virus has also been reported to occur in France (11), the United States (19,22) and Lebanon

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(13). In Taiwan, cucumber mosaic virus(CMV) (4,27), watermelon mosaic virus type-1 (WMV-1) (12), cucumber green mottle mosaic virus (CGMMV) (26) and Loofah mosaic virus (LoMV) (3) have been reported infecting cucurbitaceous plants. In 1982, we observed a disease which produced severe mosaic and rugose symptoms on leaves and distortion and malformation of fruits of cucumber plants. On the basis of symptoms, host range and serological relationships, we proposed that zucchini yellow mosaic virus is the cause of the disease (9). Recently, in screening of 54 cucumber cultivars/lines for resistance to cucurbit viruses, Yang (27) found that ZYMV is the most destructive to cucumber crops.

Lisa et al. (15) have characterized and purified ZYMV. Recently, Provvidenti et al. (19) have also purified the cytoplasmic inclusion proteins from ZYMV infected plants. However, because of the tendency of the virus to aggregate, difficulties are often encountered in purifying the virus, especially for a high yield. In comparative studies, Huang and Hseu (10) found that different ZYMV isolates greatly influenced the virus yield under same purification procedure, although these isolates displayed serological identity in SDS-immunodiffusion tests (9). This paper deals with a selected ZYMV isolate which we purified and consistently gave high yields of purified virus by the procedure of Gonsalves and Ishii with some modifications (8).

Materials and Methods

Source and maintenance of the virus isolate: The ZYMV isolate used mostly in this study referred to as ZYMV-7 (9) was originally obtained from leaves of infected cucumber plants grown in the experimental field of Taiwan Agricultural Research Institute. It had passed through three serial transfers from a local lesion host, *Chenopodium amaranticolor*, to zucchini plants. The isolate produced severe mosaic and leaf distortion on zucchini, and latent infection on *Pisum sativum* 'Alaska'. It was generally maintained and propagated on zucchini plants through this study.

Determination of virus concentration in zucchini: To determine the time required for the virus to reach highest concentration in systemically infected leaves of zucchini plants, leaves were sampled from three inoculated plants at 2-day intervals. About six discs (0.5cm in diameter) were taken from each leaf by a cork borer. Then, the leaf discs were pooled, weighed, and ground in 10 volumes of 0.05M potassium phosphate buffer, pH 7.5, (w/v). The crude extract was inoculated to two *C. amaranticolor* plants for producing local lesions. The relative virus concentration in systemically infected leaves was estimated by counting the number of lesions 10-14 days after inoculation.

Purification: The purification procedure for ZYMV was a modification of that described by Gonsalves and Ishii (8) for purifying papaya ring-spot virus. Systemically infected leaves were harvested from greenhouse-grown zucchini plants 9-10 days after inoculation with ZYMV. The leaf tissue was homogenized with a Waring blendor (Model 31BL42) in 0.25M potassium phosphate buffer (2ml/g tissue), containing 0.01M disodium ethylenediaminetetraacetate (EDTA) and 0.1% Na_2SO_3, pH 7.5. Chloroform and carbon tetrachloride, each at 0.5ml/g tissue, were added slowly as the tissues were being

ground. The homogenate was centrifuged at 5,000 rpm (Hitachi RPR-12) for 5 min, and the supernatant centrifuged again at 8,000 rpm for 15 min in same rotor. PEG (polyethylene glycol, M. W. 6,000) was added to the supernatant at the rate of 8g/100ml. The mixture was stirred for 1-2 hr and was centrifuged at 10,000g for 20 min in a Hitachi RPR-16 rotor. The resulting pellets were resuspended in 0.1M potassium phosphate buffer plus 0.01M EDTA, pH 7.5, and stirred for another hour. Before the second PEG treatment (5% PEG and 0.3M NaCl), the resuspended virus was centrifuged at 4,000-5,000 rpm for 10 min to remove the host constituents. The virus was precipitated after second PEG treatment, and the pellets were finally resuspended in 0.05M potassium phosphate buffer, pH 7.5, and stirred overnight.

Upon isopycnic centrifugation, the virus suspension was mixed with 26% cesium sulfate (w/w), or with 30% cesium chloride. Centrifugation was performed at 38,000 rpm for 22-24 hr at 6°C in a Hitachi RP-65T rotor ($w^2t = 1.28 \times 10^{12}$ rad^2/s). After centrifugation, the virus zone was collected by a small pipet. The purified virus was diluted to five times its volume with phosphate buffer, and again centrifuged at 10,000g for 10 min. The virus preparation was dialyzed overnight against 0.05M phosphate buffer to remove Cs salt for spectrophotometry, or it was given another cycle of isopycnic centrifugation.

The purity and quantity of the purified virus preparations were determined by a Hitachi spectrophotometer (Model 220S). In one trial, the absorption profile was measured at 254 nm with UV monitor (Gilson LC detector, Model 111) coupled with Gilson CRP fractionator for virus sample from the second cycle of isopycnic centrifugation in Cs_2SO_4.

Production of antiserum: All antisera to ZYMV-7 were produced in one rabbit by injecting virus purified after one cycle of isopycnic centrifugation. The rabbit was given a series of four intramuscular injections at weekly intervals, and a booster at 6 wk after the final injection. The immunized rabbit was bled weekly, starting 1 wk after the last injection. Antibodies reacting with host proteins were removed by adding 2% of bovine serum albumin (BSA) (Sigma Co.) to the sera, and usually incubating the mixture overnight at 4°C. All sera were stored in the presence of 0.02% sodium azide at −20°C.

Serology: The titer of antiera was determined either by ring interface precipitin test (2), and or by SDS-immunodiffusion test (14,20) against homologous virus. Serological reactions were determined among the following ZYMV isolates: ZYMV-1 to ZYMV-9 from Taiwan (9), and ZYMV-FL (FC1119) from Florida. In addition, WMV-1 and WMV-2 (Florida isolate) were also included for the purpose of comparisons. Both ZYMV-FL and WMV-2 were kindly provided by Dr. D. E. Purcifull, University of Florida. The agar medium for immunodiffusion tests consisted of 0.8% Noble agar, 1% sodium azide and 0.3% dodecyl sulfate sodium (SDS) (14,22). In some cases 2% bovine serum albumin was incorporated into the medium to remove the non-specific reaction (4,20,23). Crude antigens were prepared from fresh leaves of inoculated zucchini plants by grinding 1ml/g tissue in distilled water, followed by adding 1ml of 3% SDS,

and filtered through cheesecloth. Generally, the whole procedure was completed within one hour.

Results

Virus concentration in zucchini: Leaf extracts prepared from systemic leaves of inoculated zucchini plants were assayed for virus concentration by inoculation to *Chenopodium amaranticolor*. The local lesions produced on *C. amaranticolor* showed that virus content increased gradually in zucchini during the first 6 days, and then it increased rapidly. A peak virus concentration was reached in systemically infected plants about 8-10 days after inoculation under the growth chamber conditions (25°C) (Fig. 1). Therefore, leaves were usually harvested at this time and used for virus purification and serological studies.

Purification: In our earlier purification attempts, virus suspension was mixed with 30% Cs_2SO_4 for isopycnic centrifugation, the virus zone appeared at about 1.5cm below the meniscus after centrifugation for 22-24 hr using Hitachi RP-65T rotor tubes. It was close to the host green components and needed a great care to collect it by a small pipet. Later, the virus suspension was mixed with 26% Cs_2SO_4. After isopycnic run the virus zone was located just below the middle of the tube and was well separate from the green materials. The purified virus was easily collected from Cs salt gradient.

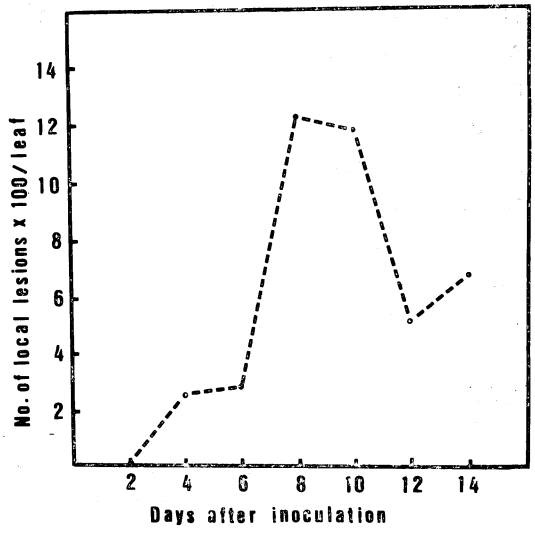


Fig. 1. Virus concentration of zucchini yellow mosaic virus in relation to the time of infection in zucchini plants under the constant temperature at 25°C The relative virus concentration was estimated by counting the local lesions produced on*Chenopodium amaranticolor*

The purity and quantity of the purified virus preparations were analyzed by spectrophotometer. Virus recovered from the first isopycnic centrifugation was found contaminated with host proteins. However, a second cycle of isopycnic centrifugation yielded highly purified virus (Fig. 2). The absorption of several separate preparations of the purified virus was lowest at 246-247 nm and highest at 260 nm, and the O. D. ratio of A260/280 ranged from 1.27 to 1.31 (uncorrected for light scattering). Assuming $E^{0.1\%}_{260}=2.4$ (21), yield of the purified virus was estimated to be 18-22mg/100g leaf tissues.

In one additional trial for comparing virus stability in cesium sulfate and cesium chloride solutions, the virus suspension was mixed with Cs salts. After isopycnic centrifugation, virus preparations from both Cs salts were inoculated to *C. amaranticolor* after dialysis. At O. $D._{259}=0.593$, the purified virus from CsCl gradient produced 1835

lesions/leaf, and at O. D.$_{260}$=0.528, that from Cs_2SO_4 gradient produced 1378 lesions/leaf. These results indicated that ZYMV-7 is stable in both Cs salts.

Serology: In ring interface precipitin tests the reciprocal titers of antiserum, taken from the third bleeding, were 2048 and 4096 against homologous antigen in crude leaf extracts and purified preparations, respectively. This antiserum reacted with healthy plant extracts at 1:4 dilution. Reacting with crude antigen in SDS-immunodiffusion gel, the antiserum had a titer of 16. There were no precipitin band formation when it was tested against healthy plant extracts. However, antisera collected from several bleedings reacted with host proteins weakly (Fig. 3, b). Generally, we added 2% BSA into the agar medium to remove the nonspecific reactions (Fig. 3, a).

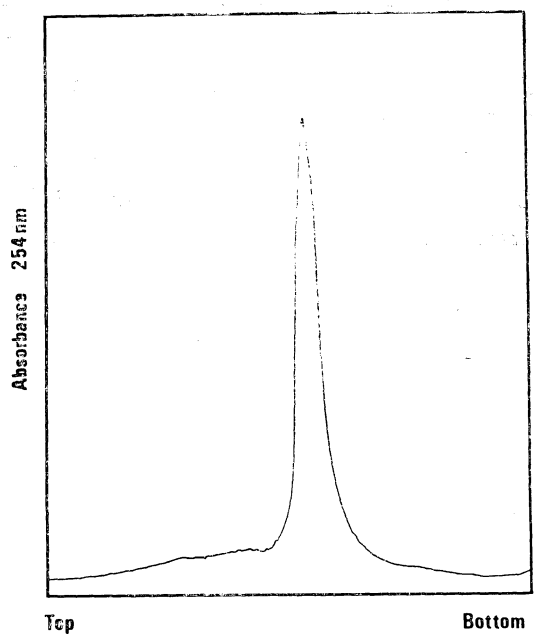


Fig. 2. Absorption profile of the purified zucchini yellow mosaic virus from the second cycle of isopycnic centrifugation in Cs_2SO_4.

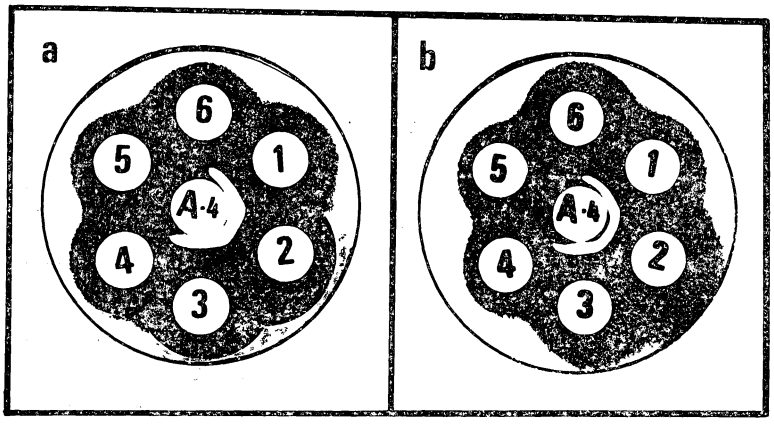


Fig. 3. Serological reactions of zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus-1 (WMV-1), and watermelon mosaic virus-2 (Florida isolate) with antiserum to ZYMV-7.
The central wells (A-4) contained antiserum to ZYMV-7 collected 4 wk after the last injection. The peripheral wells contained SDS-treated antigens: 1: ZYMV-4; 2: ZYMV-FL (Florida isolate); 3: ZYMV-7; 4: WMV-2 (Florida isolate); 5: WMV-1; 6: leaf extracts from healthy zucchini plants.
The medium for pattern (b) consisted of 0.8% Noble agar, 0.3% SDS, and 1% NaN_3, whereas medium for pattern (a) consisted of the above constituents plus 2% BSA. The non-specific reaction was observed in (b), however, it disappeared in (a).

ZYMV-1 to ZYMV-9, and ZYMV-FL appeared to be serologically identical in SDS-gel diffusion tests. Except that ZYMV-2 sometimes formed a weak precipitin line, they formed strong precipitin bands without spur reaction when tested against antiserum to ZYMV-7 (Fig. 4, a, b) or antiserum to ZYMV-Italy (Fig. 4, c) (provided by Dr. V. Lisa, Italy). There were no serological relations between ZYMV-7 and WMV-1 or WMV-2 (Florida isolate) (Fig. 3). However, an antiserum to WMV-2 obtained from Dr. D. E. Purcifull which reacted with WMV-2 Florida isolate strongly, also produced precipitin bands when tested against ZYMV-4, ZYMV-7 and ZYMV-FL (Fig. 4, d). These results indicate that ZYMV isolates from Taiwan were serologically related to certain types of WMV-2.

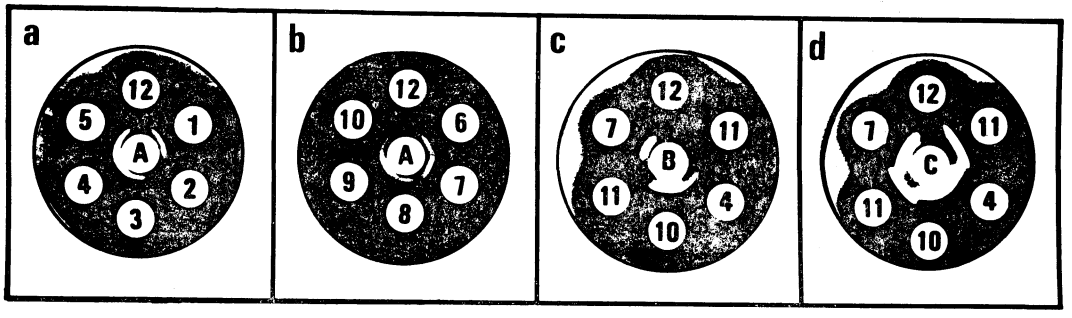


Fig. 4. Serological comparisons of zucchini yellow mosaic virus isolates and watermelon mosaic virus-2 (Florida isolate) in SDS-immunodiffusion tests.
The central wells (A) contained antiserum to ZYMV-7, (B) antiserum to ZYMV-Italy (kindly provided by Dr. V. Lisa), and (C) antiserum to WMV-2 (kindly provided by Dr. D. E. Purcifull). The peripheral wells contained SDS-treated antigens from zucchini leaves infected with: ZYMV isolates Nos. 1-9 from Taiwan; 10=ZYMV-FL (Florida isolate); 11 : WMV-2 (Florida isolate); 12=healthy zucchini squash leaves.

Discussion

Aggregation of virus particles during purification has been considered a limiting factor in obtaining higher yields of purified virus in the PVY group (8, 15, 17, 24, 25). ZYMV, a potyvirus recently reported by Lisa et al. (15), is no exception. The use of chloroform for clarification or of high molarity buffers for resuspension did not improve the yields of the virus (15). In our initial purification attempts, an isolate designated ZYMV-4 (9) was selected and the yield of purified virus was low, probably due to the losses of virus from aggregation during purification; however, when ZYMV-7 was proceeded through the same procedure, a relative high yield of purified virus was obtained, suggesting that different ZYMV isolates greatly affect the yield of virus in purification (10).

The addition of EDTA to extraction or resuspending buffers to reduce virus aggregation had been proposed (5, 8, 24, 25). Deigado-Sanchez and Grogan (5) obtained an UV absorption spectrum typical of nucleoprotein for purified virus resuspended in borate buffer plus 0.01M EDTA. Our initial experiments showed that the addition of Na-EDTA at 0.01M in purified virus preparations changed the UV spectra. It also affected the value of O. $D._{260/280}$. Therefore, while EDTA was used to prevent virus aggregation,

its effect on spectrophotometric measurement should be taken into account.

The purified virus preparations from either the first or the second isopycnic centrifugation gave an identical UV absorption curve; however, the virus preparations from one isopycnic centrifugation were still contaminated with host proteins as previously observed by others (1, 8, 24). The value of $A_{260/280}$ ranging from 1.27 to 1.31 in our studies was a little high, presumably due to contamination with host nucleic acids by the use of phosphate buffer (6, 21), but it fitted into the range of 1.2-1.37 reported for other potyviruses (7, 18).

Our ZYMV isolates and the Florida isolate ZYMV-FL appeared to belong to the same serogroup in SDS-immunodiffusion tests. They were not serologically related to WMV-1 or WMV-2 (Florida isolate). Lisa et al. (15) found that ZYMV reacted with two antisera against Italian isolates of WMV-2. Purcifull et al. (22) pointed out that antiserum to WMV-2 collected at late bleedings could reacted with ZYMV (Florida isolate 1119) antigen. In our studies, an antiserum to WMV-2, provided by Dr. D. E. Purcifull, reacted with our ZYMV isolates but formed spur reactions. This result confirmed that ZYMV isolates were serologically, but distinctly, related to certain isolates of WMV-2 as reported previously (15, 22).

Since ZYMV has not been extensively investigated in Taiwan, the collection of more ZYMV isolates from different crops and study of their serological relationships to WMV-2 are necessary. ZYMV has been reported to be the most destructive virus to cucumber plants (27); breeding programs designed to develop ZYMV-resistant varieties should evaluate cross progenies against a wide range of ZYMV isolates.

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矮南瓜黃化嵌紋病毒之純化與血清學特性之研究[1]

黃秋雄　許秀惠　趙玉珍[2]

摘　要

將 Gonsalves 及 Ishii 純化木瓜輪點病毒所使用之等密度平衡離心法 (isopycnic centrifugation)略加修改，可成功地純化矮南瓜黃化嵌紋病毒(zucchini yellow mosaic virus, ZYMV)。以接種 ZYMV-No. 7 分離株之矮南瓜爲純化材料，估計每百克病葉可獲得22毫克之純化病毒。紫外光吸收比值$A_{260/280}$約在1.27至1.31之間。所純化之病毒懸浮液適量混合輔助劑 (adjvuant) 後注射入小白兎使產生抗血清。利用界面環沈澱法，將抗血清與本病毒之粗汁液及純化之病毒反應，測得血清力價分別爲2048及4096。

本省九個ZYMV分離株及一個美國佛州ZYMV-FL分離株，以SDS-瓊脂免疫擴散法來探討各分離株血清類緣關係，結果發現所有分離株均與 ZYMV-No. 7抗血清形成明顯沈澱帶且互爲融合，證明各分離株均有相同血清類緣關係，但與 WMV-1 或 WMV-2 (佛州分離株) 無關。進一步試驗，發現本省之ZYMV 分離株與一個來自佛州大學之 WMV-2 抗血清有血清類緣關係。由是可知，本省之ZYMV 分離株異於 佛州一個 WMV-2 分離株，但與 WMV-2 部份分離株有血清類緣關係。

(關鍵字：病毒純化，血清學研究，矮南瓜黃化嵌紋病毒)。

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