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ELUCIDATION OF MECHANISM OF RESISTANCE OF TOMATO AGAINST TOBACCO MOSAIC VIRUS USING PROTOPLASTS

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ABSTRACT

Leaf protoplasts of tomato variety Marikit was successfully isolated with enzyme solution used. More protoplasts were obtained using younger leaves and leaf sections than older and whole leaves.

The mechanism of resistance to tobacco mosaic virus (TMV) of two resistant varieties, Acc. 549 and TMC 106, was elucidated using protoplasts. The resistant varieties exhibited symptomless reaction upon TMV inoculations unlike the susceptible varieties, Marikit and VC 11-1, which showed severe mosaic symptoms. The TMV-inoculated protoplasts of both susceptible varieties showed positive reaction with very intense yellow color in ELISA test. This implied a high virus concentration on the protoplasts and that the virus may have rapidly multiplied and spread from cell to cell with the leaf tissues. In the resistant varieties, protoplasts of Acc. 549 gave slight yellow color reaction in contrast with the protoplasts of TMC 106, which yielded a negative colorless reaction in the ELISA test. Using local lesion host, *Nicotiana glutinosa*, resistant variety Acc. 549 induced necrotic local lesion s from 1-wk old inoculum, while TMC 106 did not produce any lesion even from 4-wk old inoculum source. On the contrary, susceptible varieties Marikit and VC 11-1 induced necrotic lesions even from 4 wk-old inoculum source.

Key words: tomato, tobacco mosaic virus, protoplasts

INTRODUCTION

Protoplasts are cells without cell wall. They are easily isolated using mixtures of enzymes (Kassanis and White, 1974). The potential application of protoplasts in physiological and genetic studies of plants and in recombinant DNA studies in microorganisms has been early recognized. In studying plant viruses, protoplasts offer some significant advantage over whole organism or an organ. This system was proven useful for cytological (Otsuki *et al.*, 1972a), physiological (Otsuki *et al.*, 1972b), and biological studies (Sakai and Takebe, 1972).

Elucidation of mechanism of disease resistance can either be explained morphologically or biochemically which is easily understood with diseases due to bacteria, nematodes or fungi. However, viruses have a unique system of entry into the plant cells. The use of plant protoplasts in the study of plant viruses was initiated by Cocking (1972) and Zaitlin and Beachy,

(1974). The multiplication and spread of cucumber mosaic virus viruses inside the protoplasts of susceptible melon variety were noted (Hirai and Amemiya, 1989).

Several assays to test the infectivity of the virus in the protoplasts have been mentioned These include local lesion host (Takebe *et al.*, 1968), enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977) and fluorescent antibody staining technique (Otsuki and Takebe, 1973).

In the Philippines, no attempt has been made to isolate protoplasts of tomato and to elucidate the mechanism of resistance to tomato against tobacco mosaic virus using protoplasts.

This study aims to (a) identify resistant and susceptible varieties of tomato, (b) isolate and optimize conditions for isolation of tomato protoplasts, (c) isolate purify and propagate tobacco mosaic virus (TMV) and, (d) assay the infectivity of TMV-infected leaves and protoplasts using ELISA and local lesion hosts.

METHOLODOGY

Identification of Susceptible and Resistant Sources

Various germplasm of tomato were evaluated for resistance to TMV. The disease screening technique and rating scales developed being used at the Institute of Plant breeding, UPLB were followed. The identified resistant as well as susceptible varieties were used in the study. The symptoms developed on these varieties were described.

Collection, Purification and Propagation of the TMV

An important virus isolate found infecting tomato was used in the study. TMV was propagated in tomato var. Marikit. The virus was purified according to the modified procedure of Gooding and Hebert (1967). Extraction was done in 0.1 M phosphate buffer, pH 7.2 and 1% mercaptoethanol. Clarification of virus sap was done with 8% butanol (v/v) and low speed centrifugation (5,000 g for 15 min). The virus was precipitated with 6% polyethylene glycol (PEG) and 4% NaCl and low speed centrifugation. Resulting supernatant was further purified by high speed centrifugation (25,000 rpm for 2 hr) and low speed centrifugation (10,000 g for 15 min). Infected plants were kept and maintained in the greenhouse

Antiserum Production

Antiserum to TMV was raised in 2½ mo-old rabbit by giving a single intravenous injection containing 1 mg/ml TMV. After 1 wk rest period, the 2nd, 3rd and 4th implied via intramuscularly route were given at 200 ug, 200 g and 100 ug, respectively, for 3 wk. The rabbits were allowed to rest for another 2 wk until a final booster shot at 500 ug was given. The final bleeding was performed 7 to 10 days after the booster shot.

Isolation of Protoplasts

Seeds of susceptible tomato variety were planted in pots and properly maintained in the greenhouse. Healthy leaves were collected from 3 to 4 wk-old plants. Leaves were taken early in morning, washed thrice with sterile distilled water and blotted dry using sterile filter paper.

Leaves were treated either as small lesions (1-2 mm^2) or as whole. Leaves were cut gently into small sections using a sharp, fame-sterilized scalpel blade before placing in enzyme solution. The whole leaf, on the other hand, was cut slightly into narrow strip with flame-sterilized, sharp scalpel blade to expose the tissue and allowing the enzyme solution to penetrate the cut tissues by applying low vacuum for 5 min. The effect of the age of leaf on protoplast yield was also determined. Younger leaves were taken from

the upper most part of the plant, the shoots while older leaves were taken from the lower branches of the plant.

Different enzyme solutions with varying concentration of enzymes such as cellulase 'Onosuka' R 10 (ca. 11,000 units/g) and macerozyme R 10 (ca. 450 units/g) from Yakult Honsha Co., Ltd., Japan and pectinase (EC 3,2,1,1.5) from Sigma Chemicals Corporation, were used. Potassium dextran sulfate at 0.05% was obtained from Wako Pure Chemical Industries Ltd., Japan. The percentage of the enzymes varied from 1.0 to 3.0% for cellulase and 0.3 to 0.5% for macerozyme. Manitol at 0.5M to 0.75M was used. In all the tests, one gram (fresh weight) of leaf samples was placed in 20 ml enzyme solution contained in clean Erlenmeyer flask. The solution with the samples was then placed on a rotary shaker (about 60 excursions per min). Protoplast yield was monitored on an hourly basis by decanting one drop of the solution using clean Pasteur pipet and examining under the microscope. The number of protoplasts was counted using haemacytometer.

Inoculation of TMV

After isolation, protoplasts were washed 3 times with 0.6M Mannitol, pH .7. Protoplasts were then mixed with the TMV. Resistant and susceptible plants were likewise mechanically inoculated with the pure culture of TMV and infected leaves harvested after 1, 2, 3, and 4 wk from inoculation. Tissues from these plants were used as source of inoculum in the subsequent inoculations to local lesion host.

Assay of Infectivity

Infectivity of TMV on leaves of resistant and susceptible tomato varieties as done using local lesion host, *Nicotiana glutinosa* and by ELISA. Lesions appearing on the half leaf assay host were counted/estimated. The ELISA test was performed on inoculated susceptible and resistant varieties harvested after 1, 2, 3 and 4 wk from inoculation. Similarly, protoplasts isolated from these tomato varieties were also assayed using this method.

.RESULTS AND DISCUSSION

Identification of Resistant and Susceptible Sources

Susceptible tomato varieties, Marikit ad VC 11-1 and resistant, Acc 49 and TMC 106 were selected from various tomato germplasm and used in the study.

Virus Identification and Symptomatology

Virus isolates obtained belong to tobamovirus group possessing rigid rod particles under the electron microscope. This yellow isolate from tomato induced systemic mosaic symptoms to *Nicotiana tabacum* and reacted positively against the TMV antiserum in ELISA tests. Both the resistant tomato entries (Acc 549 and TMC 106) exhibited symptomless appearance with TMV inoculation while both susceptible (Marikit and VC 11-1) produced severe mosaic symptoms (Fig 1).

Isolation of Leaf Protoplasts

Enzyme solution that provided good results contained the following 05% cellulase 'Onozuka' R 10, 0.5% macerozyme R10, 0.75M Mannitol, 0.05% potassium dextran sulfate, pH 7.5. Tomato protoplasts were successfully isolated from leaf tissues. They were perfectly round and bright green (Fig. 2). Younger leaf tissues yielded more protoplasts than older leaves (Fig. 3). Mean number of protoplasts was 163 x 10^3 per ml on older tissues. The protoplasts from young and old leaves started to be released on the second hour of incubation and increased as the length of time increased. Younger leaves producing more protoplasts could be due to

the softness of the tissues that enzyme mixture can easily penetrate the mesophyll cells.

Small leaf sections yielded more protoplasts than whole leaf (Fig. 4). Protoplasts from leaf sections were obtained on the second hour of incubation, while release of protoplasts from whole leaf was noted on third hour of incubation. This could be attributed to the availability of cut surfaces that are exposed to the enzyme solution. In both experiments, it was observed that more debris was present in the solution containing the leaf section than on whole leaf.

Assay of Infectivity

The two resistant (Acc 549 and TMC 106) and two susceptible tomato entries, Marikit and VC 1101 inoculated with pure cultures of TMV were harvested staggered 1, 2, 3 and 4 wk post-inoculation which then served as the sources of inocula to *N. glutinosa*. The TMV from 1 wk-old Acc 549 induced 2.5 necrotic lesions to the assay host while the 2, 3 and 4 w-old sources of inocula did not cause any lesion. The other resistant entry, TMC 106 on the other hand did not elicit any lesion to the assay host using the different sources of inocula harvested from 1 to 4 wk post-inoculation. Such results suggest that TMV has been able to enter the resistant tomato, Acc 549 only for a certain period of time (one wk) but was not able to multiply or spread from cell to cell within the plant. The virus particles were localized within the small leaf area as evidenced by necrotic local lesions on the assay host. Negative reactions of both the local lesion host and ELISA results of he assay from the second to fourth week-old inocula revealed that the virus might have already died inside the cell.

In the other resistant tomato, TMC1 06, the virus was not able to enter the plant. On the other hand, the TMV was able to invade and progress in the cells of both susceptible varieties, Marikit and VC 11-1 as evidenced by the several lesions ranging from 7 to 8.5 after the first week and more than 15 lesions on the third week. The number of lesions could hardly be counted since the lesions coalesced after the third week. Results of ELISA tests conformed to these results giving positive reaction only to both susceptible varieties and to 1 wk-old Acc 549 (Table 1).

Isolated Protoplasts

The isolated protoplasts of both susceptible varieties showed positive reaction with an intense yellow color suggesting high virus concentration on the protoplasts (Table 2). This implied that the virus had multiplied and spread from cell to cell within the leaf tissue. In the resistant entries, protoplasts of Acc 549 gave only a slight yellow color reaction to the antibody suggesting much lower virus concentration while the TMC 106 protoplasts produced negative reaction.

These findings showed that TMV was able to multiply and spread in the susceptible varieties but not in the TMC 106 resistant entry. In case of Acc 549, where only slight reaction in ELISA test was observed suggesting that the virus was able to enter the plant only in low concentration and did not multiply in the plant. This conformed with our infectivity assay findings for Acc 549 (Table 1) giving lesions only for 1 wk-old source but none on 2 to 4 wk-old sources of inocula. These findings conform with the result of Hirai and Amemiya (1989). They found that cucumber mosaic virus infected cells of susceptible melon variety, Earl, increased with time after inoculation, while in resistant variety, Kohimeuri, virus infected cells were localized within a small area.

Based on these results, two types of resistance were exhibited by the tomato entries. TMC 106 is immune to TMV while Acc 549 displayed 'hypersensitive type' of resistance. The virus in Acc 549 was able to enter the plant only for short period of time, became localized in small areas in the leaf tissue but later on died as evidenced by the

formation of necrotic local lesions.

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Table 1. Results of infectivity assays to tobacco mosaic virus (TMV) of two susceptible and two resistant tomato varieties

VARIETY	REACTION TO TMV	AGE OF INOCULUM[1]	LOCAL LESION TEST[2]	ELISA TEST[3]
Marikit	Susceptible	1	7.0	+++
		2	12.0	+++
		3	>15 (coalesced)	+++
		4	coalesced	+++
VC 11-1	Susceptible	1	8.5	+++
		2	13.0	+++
		3	>15 (coalesced)	+++
		4	coalesced	+++
Acc 549	Resistant	1	2.5	+
		2	0	-
		3	0	-
		4	0	-
TMC 106	Resistant	1	0	-
		2	0	-
		3	0	-
		4	0	-

[1] Number of weeks harvested from TMV inoculation.
[2] Number of lesions on *Nicotiana glutinosa.*
[3] Reaction to TMV antiserum, - negative to TMV, + positive to TMV.

Table 2. Results of ELISA test to the protoplasts from tobacco mosaic virus (TMV)-infected susceptible and resistant tomato varieties

VARIETY	REACTION	VIRUS REACTION[1]
Marikit	Susceptible	+++
VC 11-1	Susceptible	+++
Acc 549	Resistant	+
TMC 106	Resistant	-

[1] Reaction to TMV antiserum: - negative to TMV, + positive to TMV.

Figure 1. Reaction of resistant, Acc 549 (left) and susceptible, Marikit (right) tomato varieties to tobacco mosaic virus.

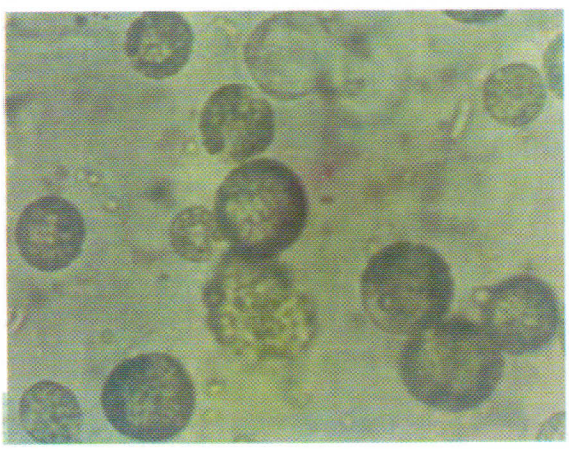

Figure 2. Isolated protoplasts of tomato variety Marikit.