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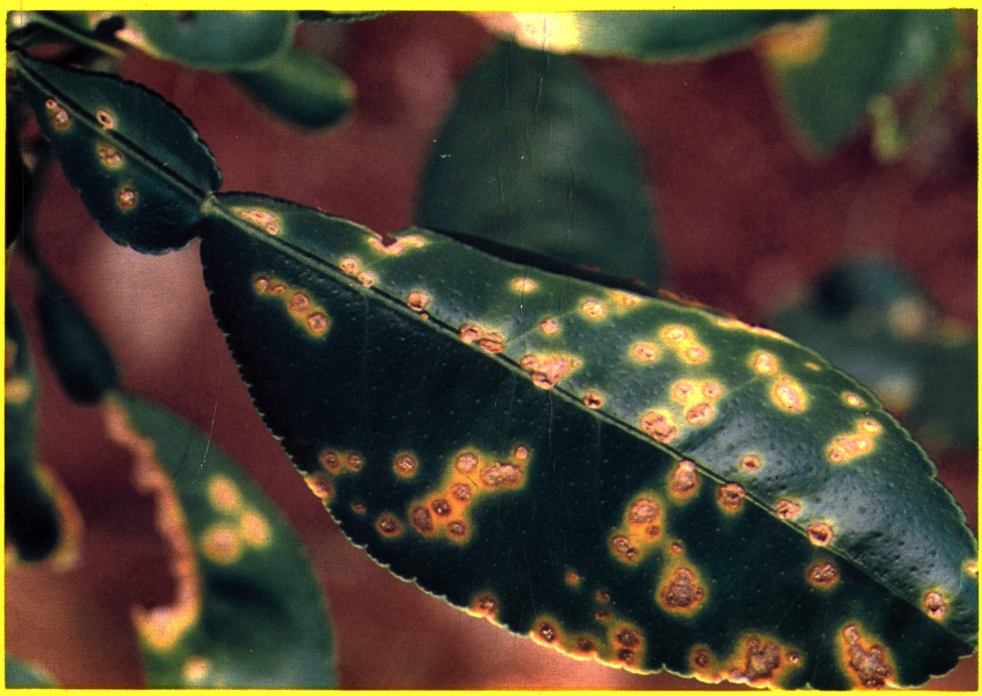
1. THE SEROLOGICAL DIAGNOSIS OF CITRUS CANKER



J. S. JAYASEKARA AND T. SUETSUGU

NATIONAL PLANT QUARANTINE SERVICES PROJECT
JAPAN INTERNATIONAL COOPERATION AGENCY

KATUNAYAKE, SRI LANKA

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To pay tribute in memory of late Mr. Tetsuo Suetsugu

Mr. Suetsugu was a JICA expert on Plant Pathology Inspection Technology at the National Plant Quarantine Services Project in Sri Lanka.

Since the project has started, he has made great efforts to establish and improve the Pathological work at the National Plant Quarantine Service of Sri Lanka, and his contributions to the project were invaluable.

Unfortunately, he passed away after a brief yet serious illness, at a moment his widely admired service at the N.P.Q.S. was at its best, making a great loss to the project.

Here we published a series of his achievements during his service to the project, and trust you may find this paper interesting and useful.

Yasuharu Ikegami
Team Leader
National Plant Quarantine Services Project
Japan International Cooperation Agency

March, 1998

STUDIES ON THE DIAGNOSIS OF *XANTHOMONAS CAMPESTRIS* (PAMMEL) DOWSON PV. *CITRI* (HASSE) DYE 1978

I. The serological diagnosis of citrus cankers

J. S. Jayasekara* and T. Suetsugu**

ABSTRACT

Bacterial isolate obtained from diseased lemon fruits showing brownish to blackish cankers mostly 3-8 mm in diameter on their surface coincided in their pathogenicity, bacteriological and serological characteristics with *Xanthomonas campestris* (Pammel) Dowson pv. *citri* (Hasse) Dye 1978. The pathogen showed distinct pathogenicity to lemon, lime and orange fruits when tested by needle prick inoculation. It was an aerobic, Gram negative bacterium which did not give rise to soft rot in potato tubers. Colonies developed on PSA medium were yellowish white, round, raised, convex, smooth and glistening. Fluorescent pigments were not produced on medium B of King et al., but gelatin was liquefied, and Aesculin was hydrolyzed. Living immunoantigen was used in the production of antisera. In the serological studies by slide-agglutination test and agar gel-diffusion test, the pathogen consistently showed specific reaction with this antiserum and with homologus antiserum of *X. campestris* pv. *citri* obtained from Japan showing that the bacterium is similar to Japanese isolate.

Hence, the antiserum produced in this study can be used in the serological diagnosis of *Xanhomonas campestris* pv. *citri* in the quarantine inspection for citrus canker in Sri Lanka.

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** National Plant Quarantine Services Project

INTRODUCTION

Xanthomonas campestris pv. *citri* causes cankers on citrus plants. The disease occurs on all above ground parts, leaves, fruits, twigs and branches of plants. Young and tender parts are most prone to attack. Symptoms are best observed on leaves and fruits. On leaves, the lesions appear at first as oily-looking translucent spots usually on the lower surface of leaves, later becoming raised with a brownish corky appearance with ruptured surface, frequently depressed in the middle and surrounded by a yellow halo (Fig. 1). In severe attack conditions, defoliation may occur. Lesions on fruits may be large with deep cracks which results in a low demand in price (Fig. 2).

Citrus canker has been recorded for over 75 years in Sri Lanka. It was first recorded, in 1922 on the leaves of four-years-old lime trees in experimental plots of Anuradhapura experiment station (present Agriculture Research Station, Maha Illuppallama). Since then, it has become an important disease of citrus cultivation in Sri Lanka. It is one of the diseases which caused the declining of citrus cultivation in Sri Lanka.

Among citrus, lime is the most susceptible for this disease, while orange and lemon are less susceptible. Hence canker is more prevalent where lime is grown, particularly in wet zone of Sri Lanka.

The damage caused by this pathogen on citrus cultivation recognized the importance of studying accurate and quick diagnostic techniques for identification of different strains of *X. campestris* pv. *citri* present in Sri Lanka.

In addition to morphological informations, physiological and biochemical activities of some strains of *X. campestris* pv. *citri* were also studied.

ISOLATION OF PATHOGENIC BACTERIA

Materials: Lemon fruits with cankers were collected from Nattandiya. Canker lesions on the rind showed brownish to blackish spots, varying in size, generally 2-4 mm diameter (Fig. 3). Studies were done at National Plant Quarantine Service at Katunayake.

Isolation of pathogenic bacteria: A small tissue from the advanced portion of the lesion was removed using a sterile scalpel. The tissue was surface sterilized by dipping in 80% alcohol for 2-3 seconds, followed by 1% sodium hypochlorite solution for 2-3 minutes. After thoroughly rinsing in sterile water, the tissue was homogenized in 1 ml sterile water using a sterile mortar and pestle. From this homogenate, a wire loop was streaked as the inoculum onto Potato Semi-Synthesis Dextrose Agar medium (PSA) (Potato, 200g; $Ca(NO_3)_2$, 0.5g; Na_2HPO_4, 2.0g; Peptone, 5.0g; Dextrose, 15.0g; Agar, 15.0g; Water, 1,000 ml) and onto selective medium for *Xanthomonas* (Cellobiose, 10g; K_2HPO_4, 3g; NaH_2PO_4, 1g; $MgSO_4 \cdot 7H_2O$, 0.3g; Agar, 15g; Water, 1,000 ml., Kado and Heskett, 1970). The cultures were incubated at 25°C. Bacterial colonies were visible after 48-72 hours of inoculation. Better growth of *X. campestris* pv. *citri* was observed on PSA medium than on the selective medium. Bacteria in a yellowish colony was tested by slide-agglutination test using antiserum for *X. campestris* pv. *citri* obtained from Japan. The bacteria showing positive reaction to that antiserum were isolated and sub-cultured in PSA slants. Pure bacterial cultures were maintained in PSA slants.

Pathogenicity test: Bacterial isolate grown on PSA medium in test tubes and incubated at 25°C for 72 hours were re-inoculate on to orange, lemon and lime seedlings and herbaceous plants such as *Vigna sinensis, Nicotiana bentamina, Cucumis sativus, Tetragonia expansa, Chenopodium amaranticolor* and *Clitoria ternatea.* Healthy, young seedlings with 3-4 leaves test plants were selected for re-inoculation. Upper surfaces of young leaves were pricked with a sterilized needle in several locations and the inoculum was spread on leaves. Control plants were also pricked similarly and sprayed only with distilled water. The plants were covered with polythene bags and kept under observation at 28°C for 3 days in a growth chamber.

Growth media test: Bacteria were re-isolated from experimentally inoculated plants and grown in pure cultures on PSA medium. Their cultural disposition was compared with the pure culture of original bacteria on PSA medium.

PRODUCTION OF ANTISERUM AND SEROLOGICAL TEST

Production of antiserum: The immunization programme was carried out at Medical Research Institute in Colombo. A live bacterial cell suspension (10^{8-9} cells/ml) in sterilized saline (0.85% NaCl) was made from a pure culture in PSA medium incubated at 25°C for 72 hours was injected into rabbits (breed New Zealand white, weighing about 2 kg). Two procedures were adopted in the production of antiserum. In one procedure, the suspension of live bacterial cells were injected four times at one week intervals into the marginal ear vein of rabbits using sterilized disposable syringe with a needle of gauge 22 (Fig. 6). A primary injection of 0.5 ml and three boosters of 1 ml each were made. One week after the final injection, antiserum was collected. In the second procedure, one week after the primary intravenous injection of 0.5 ml of the antigen, 1 ml of a mixture of bacterial suspension (10^{8-9} cells/ml) and a solution of Freund's complete adjuvant (1:1 V/V) was injected only once into two rabbits intramuscularly. After one month, antiserum was collected.

Antiserum was obtained by gently taking blood from the animal's heart into sterile test tubes (Fig. 7).

The blood was allowed to clot at room temperature and centrifuged at 10,000 rpm for 10 minutes to separate the insoluble material. The antiserum was stored at 4°C with a preservative containing 0.1% NaN_3. The antiserum titer was checked by slide agglutination test.

Slide agglutination test: A two fold dilution series of antiserums in sterilized saline from 1/2 to 1/8192 was prepared. Each diluted level of antiserum and few drops of the bacteria cell suspension (10^{8-9} cells/ml) made from a pure culture incubated at 25°C for 48-72 hr. in sterilized saline (0.85% NaCl) were mixed in equal proportions on a clean microscopic slide (Fig. 8). Visual observations were made to find the highest ratio of dilution at which agglutination was still detected by comparing with antigen/saline control for each dilution level. Reciprocal of this dilution is the titer of antiserum.

Immunodiffusion Test: The agar gel-diffusion test was done in 0.8% agar dissolved in distilled water containing 0.1% NaN_3. First a Petri dish (6 cm diameter) was filled with

0.5 ml of agar. Next, metal cups (78 mm x 98 mm), were used to make a central well and six peripheral wells of equal sizes at equidistant (4 mm apart) on the agar plate. This was followed by pouring 7 ml agar into the plate. After solidification of agar the cups were pulled out of the plate. Central well was filled with antigen (0.2 ml, 10^{8-9} cells/ml) and 1st well of peripheral wells with control saline (0.2 ml, 0.85% NaCl). The remaining wells were filled with prepared antiserum and with antiserum of *X. campestris* pv. *citri* from Japan (0.2 ml/well) alternatively (Fig. 9). The plates were kept in high humidity for 1-3 days at 25°C.

RESULTS AND DISCUSSION

It was difficult to distinguish *X. campestris* pv. *citri* from other similar bacteria from citrus fruits, at the time of isolation. Colonies developed on PSA medium were yellowish, high, convex, with smooth glistening surface, circular with smooth edge and mucoid (Fig. 5). Isolated bacteria were Gram-negative, aerobic, rod-shaped and had a single polar flagellum. The thermal death point is between 50-55°C. The isolate which showed positive reaction in the serological tests with antiserum of *X. campestris* pv. *citri* Japnese strain, was used as the check isolation in this study. From the immunological tests, three strains (A, B and K7) were detected.

Pathogenicity tests confirmed the identification of bacterium. About two weeks after inoculation, small brown spots appeared on the inoculated leaves, and these spots subsequently developed into cankers (Fig. 4), similar to the ones observed on the naturally infected leaves. The herbaceous plants did not show symptoms (Fig. 10). Isolations from artificially inoculated leaves yielded bacterial colonies similar to those used for inoculations.

Results of growth and physiological tests (Table 1) coincided with the characteristics of *X. campestris* pv. *citri* (Holt et al., 1994).

According to the slide agglutination test, antiserum of strain A had a titre of 1/4096 and that of strain B had a titre of 1/2048. In the agar gel diffusion test the pathogen (strains A, B and K7) gave white precipitation bands with antiserum.

The pathogen *X. campestris* pv. *citri* (strains A, B and K7) consistently showed specific reaction with its homologus antiserum and showed to have common antigen properties with each other (Fig. 9).

More importantly, the antiserum developed for those strains can be used for serological studies for quicker and accurate recognition and identification of the strains of *X. campestris* pv. *citri* occurring in Sri Lanka.

Furthermore, serological diagnosis can be completed in several hours instead of several days if cultures are made. The techniques are sensitive, easy and relatively inexpensive. Quick and sensitive diagnostic tool is important in taking informed decisions on plant quarantine inspection.

It is concluded that the method of serological diagnosis can be adopted with regard to the diagnosis of *Xanthomonas campestris* pv. *citri* in the quarantine inspections.

ACKNOWLEDGEMENTS

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We sincerely express our profound thanks to Dr. H. Watanabe (JICA Expert), Dr. S. Jayasekara, Dr. M. G. Thammitiyagoda and Mr. W. G. S. Sisira Kumara of Medical Research Institute, Colombo for providing necessary facilities and valuable advice during in the production of antisera.

We would also like to thank Mr. R. S. Y. de Silva and Mr. W. G. S. Perera of Division of Plant Pathology, National Plant Quarantine Service for their kind assistance extended throughout the course of study.

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Physiological Tests

Table 1. The physiological characteristics of the isolates.

Test	Reaction
Oxygen requirement	aerobic
Hydrolysis of	
starch	positive (Fig. 14)
aesculin	positive (Fig. 12)
Tween 80	positive
Production of	
fluorescent pigment	negative
indole	negative
hydrogen sulfide	positive
ammonium	positive
Gelatin liquification	positive (Fig. 13)
Nitrate reduction	negative
Fermentation of glucose	oxidative (Fig. 15)
Catalase activity	positive (Fig. 16)
Utilization of asparagine	negative
Oxidase activity	negative
Urease production	negative
Potato soft rot test	Did not rot (Fig. 11)

Fig. 1 Lemon tree damaged by *Xanthomonas campestris* pv. *citri.*

Fig. 2 Citrus canker on lemon fruits. Isolate A bacterium.

Fig. 3 Citrus canker caused by *X. campestris* pv. *citri* on the leaves of *Citrus grandis.*

Fig. 4 Canker produced on citrus seedling after inoculation with isolate A.

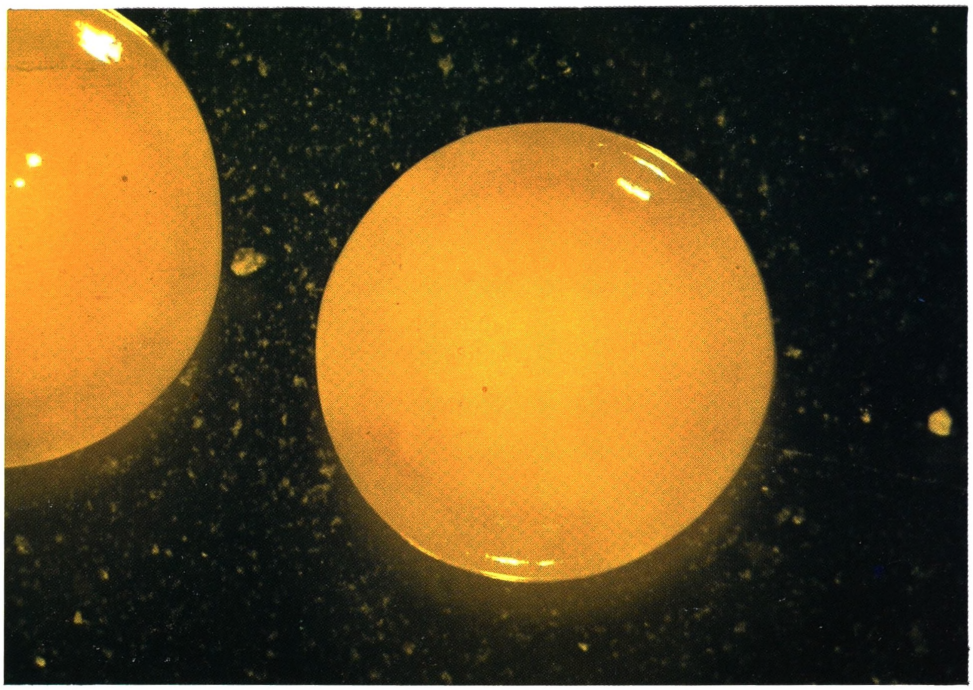


Fig. 5 Yellow colony of isolate A on PSA medium.

Fig. 6 Production of antiserum for *X. Campestris* pv. *citri* by intravenous injection into rabbit.

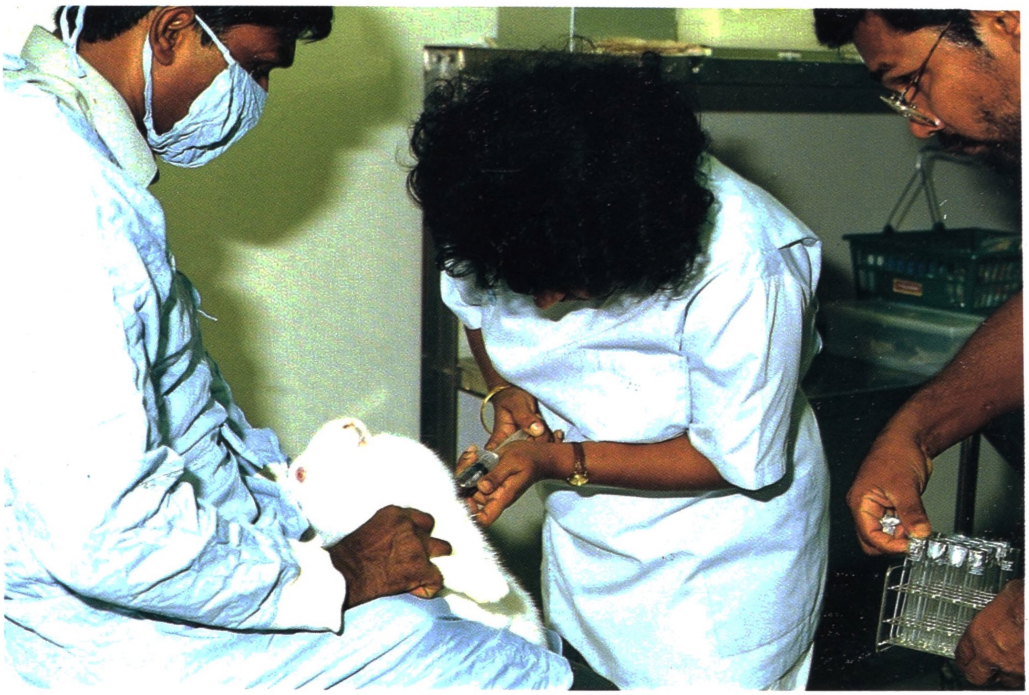


Fig. 7 Withdrawal of blood from rabbit's heart to obtain the antiserum.

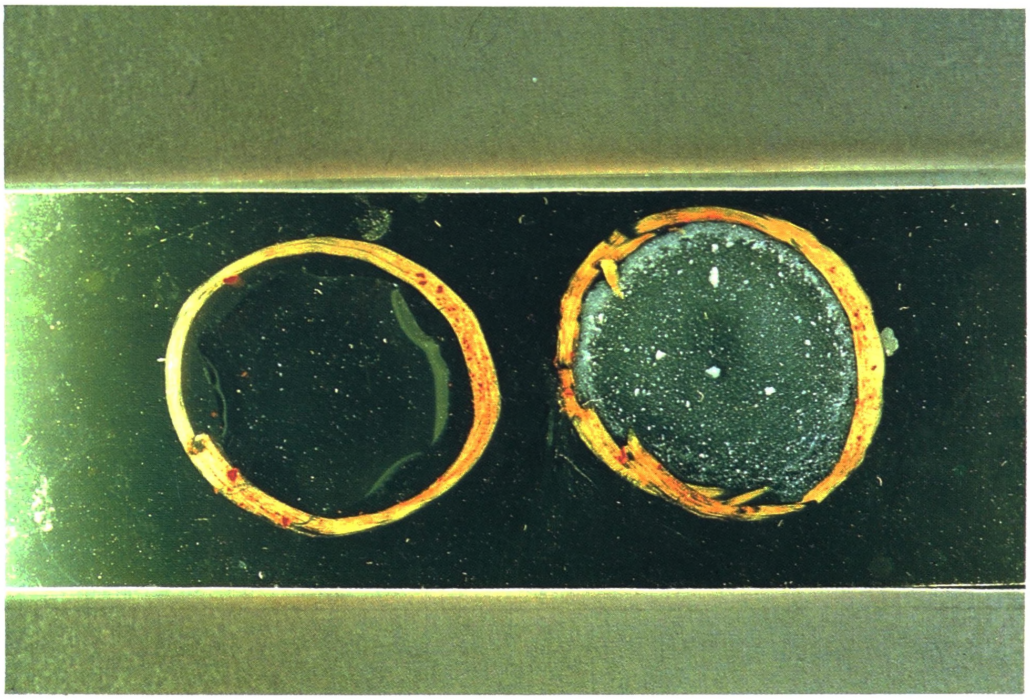


Fig. 8 Precipitation by slide-agglutination test.

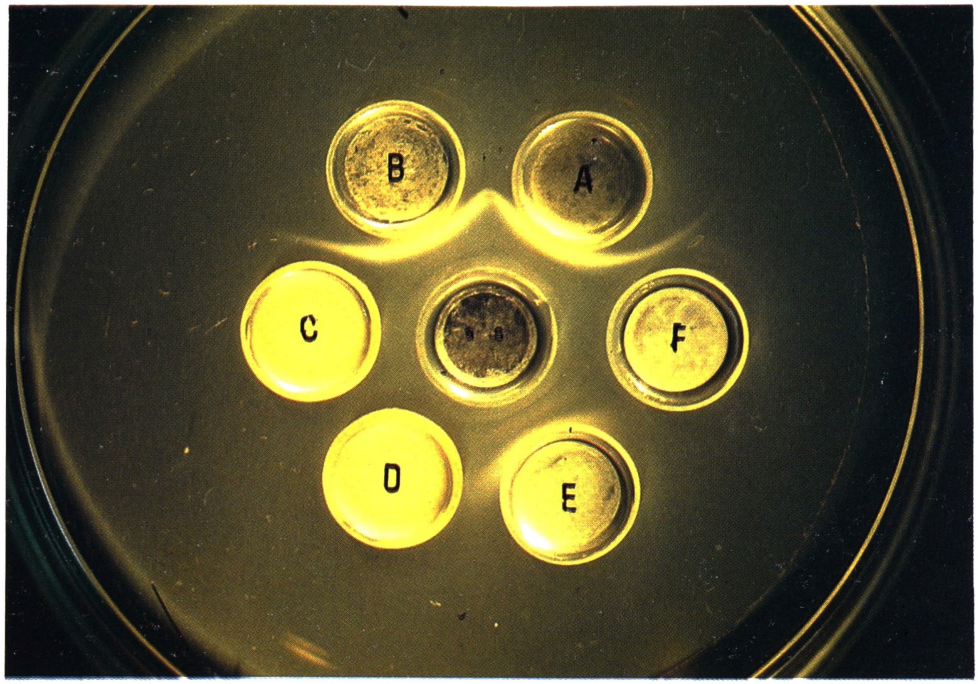


Fig. 9 Serological precipitation lines on agar-gel-diffusion for identification of *X. campestris* pv. *citri.*
A: A bacterium isolate from lemon
B: B bacterium isolate from lemon
C: bacterium isolate from lemon
D: bacterium isolate from lemon
E: C bacterium isolate from lemon
F: saline
as: antiserum of *X. campestris* pv. *citri,* isolate A

Fig. 10 Pathogenicity test: *Phaseolus vulgaris* cv. "Kentucky Wonder" unsusceptible to isolate A.

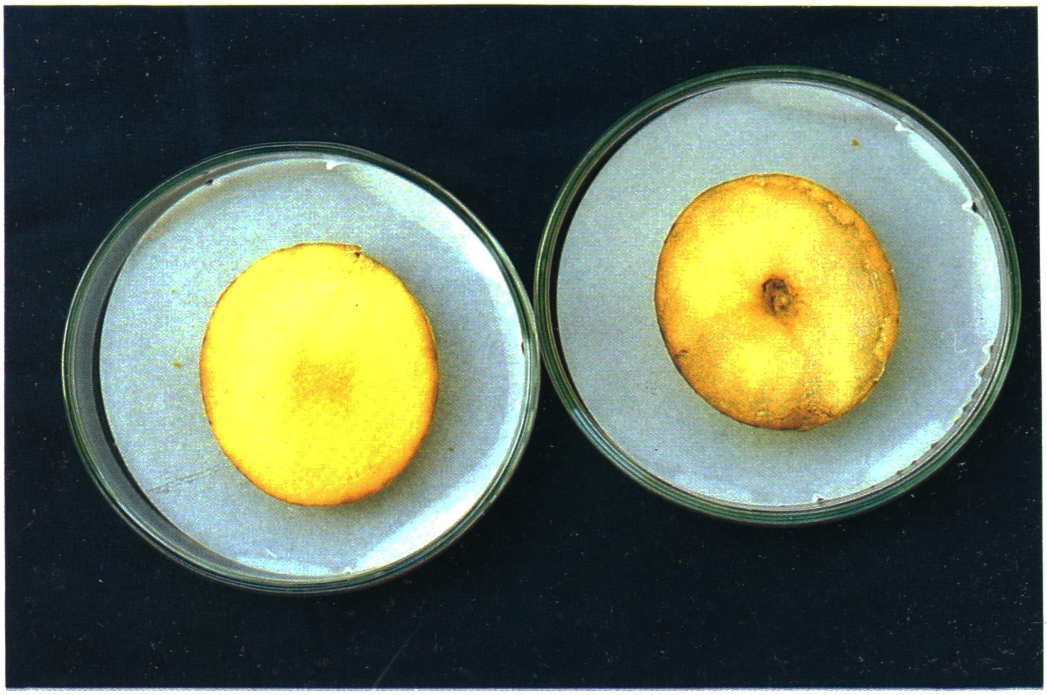


Fig. 11 Potato soft rot test: No rotting. Only damage.

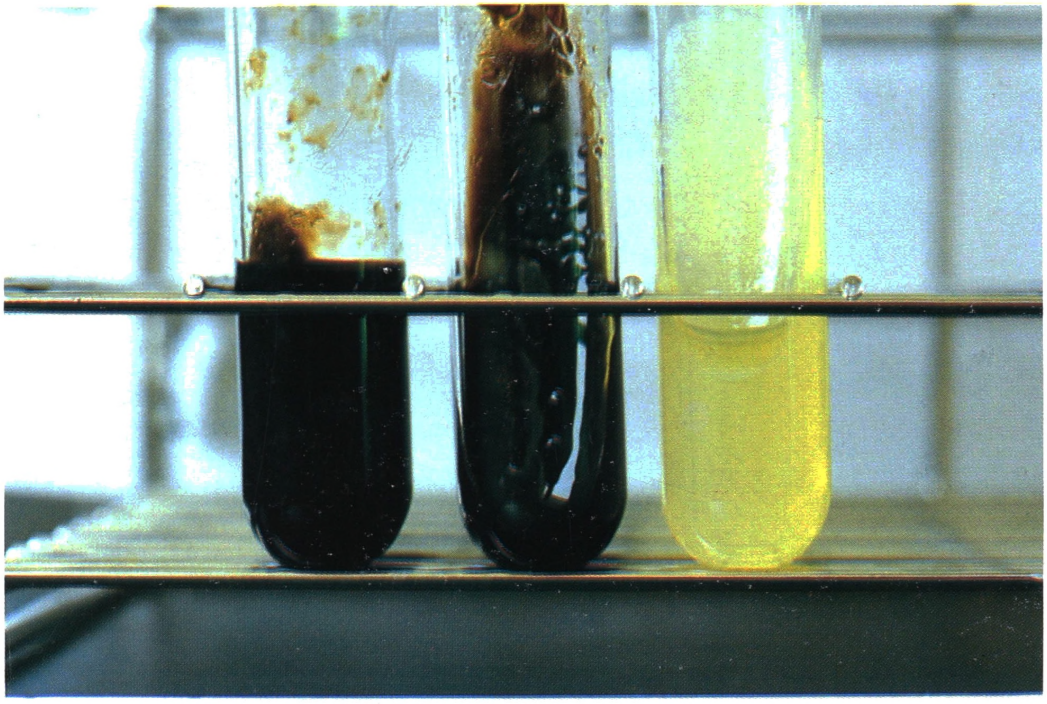


Fig. 12 Formation of a blackening pigment during hydrolysis of aesculin by isolate A.

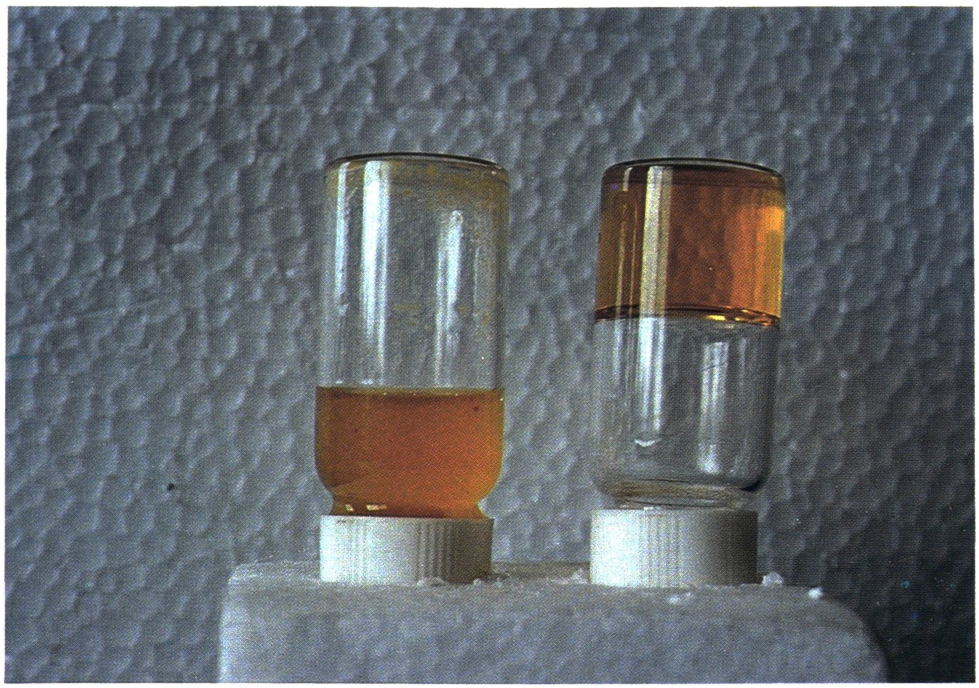


Fig. 13 Gelatin liquefaction by isolate A.

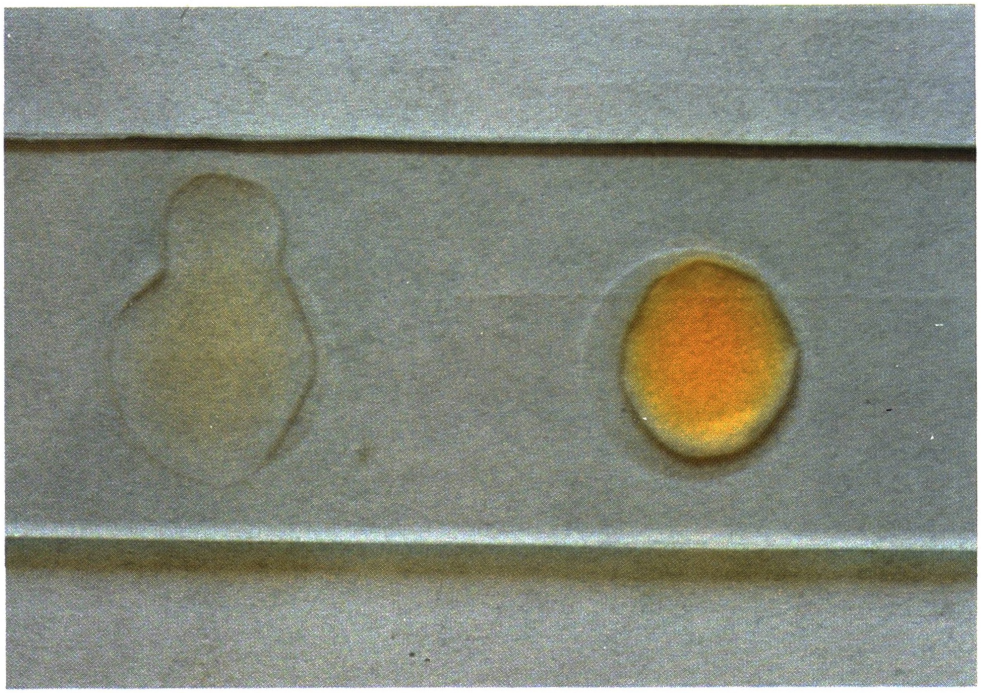


Fig. 14 Partial hydrolysis of starch by isolate A.

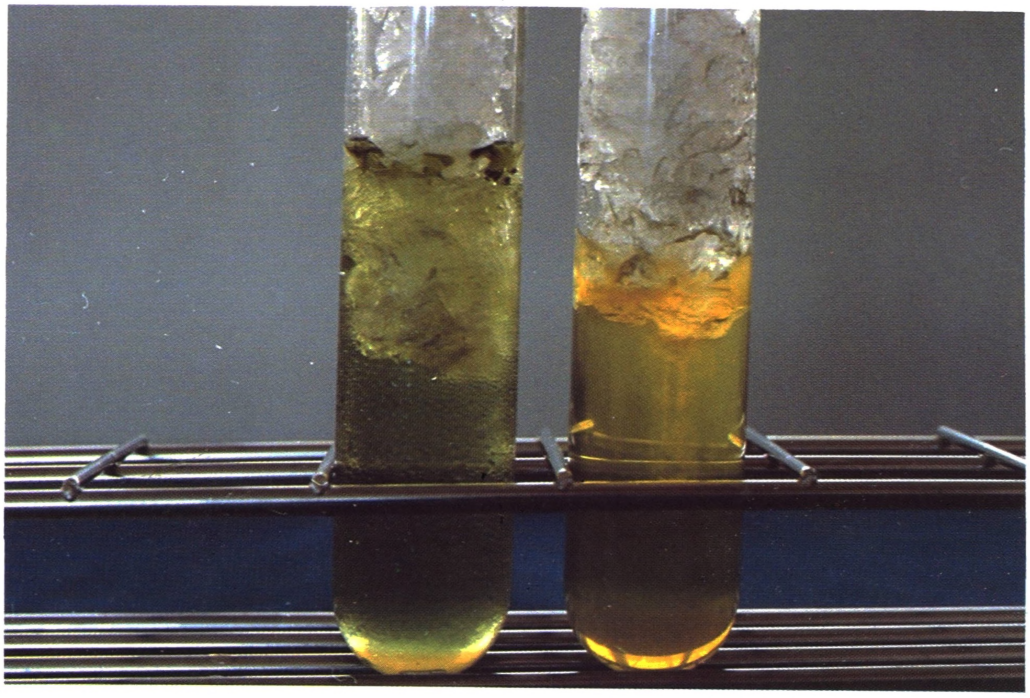


Fig. 15 Yellow coloration of Oxidative fermentation of glucose test.

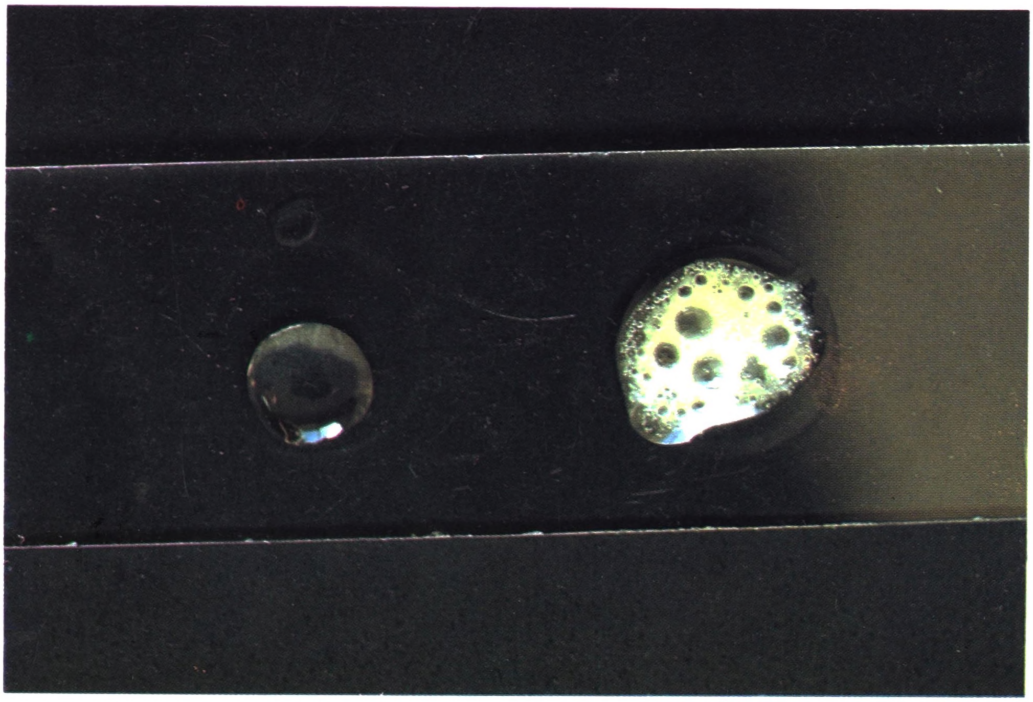
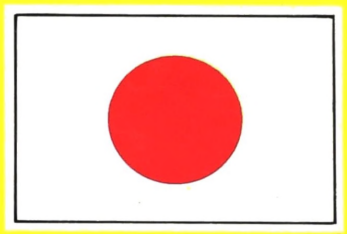


Fig. 16 Production of gas bubbles in catalase test.



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